

Fire Blight

The Disease and
its Causative Agent,
Erwinia amylovora



Edited by J.L. Vanneste



CABI Publishing

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Edited by

Joël L. Vanneste

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CABI Publishing

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Preface

This book is about fire blight, the most devastating bacterial disease of apples and pears. It is the first multi-authored book on fire blight, written by 25 international experts, who critically reviewed the literature and shared their knowledge and their experience on the disease, its causal agent and methods of control. Writing such a book was beyond the expertise of any one individual. It represents the most up-to-date information available, written by some of the most influential scientists working on fire blight today. Most chapters contain information unpublished so far. It is divided into three sections. The first section is about the disease: its epidemiology, its worldwide distribution and its economic importance, the host range of the pathogen and how it migrates and perhaps survives in the tissues. The second section is about the causal agent, *Erwinia amylovora*: its general characteristics as a member of the Enterobacteriaceae, but also the weapons it uses to cause disease: amylovoran, harpin, avirulence factors and siderophores. In the third section, the authors address the difficult problems of fire blight control. They look at chemical control, problems associated with streptomycin resistance, potential and limitations of traditional breeding and transgenic plants, risk assessment systems and models, biological control and finally integrated management strategies for the control of fire blight in orchards and in nurseries.

A reference book on fire blight has long been overdue. Since *Fire Blight: A Bacterial Disease of Rosaceous Plants*, written by Tom van der Zwet and Harry Keil in 1978, few review papers have dealt directly with fire blight. Furthermore, with few exceptions, they are restricted to a limited aspect of this disease. So, in spite of an impressive amount of work and an impressive amount of literature on fire blight, the newcomer or the interested scientist has nowhere to go to get a complete and up-to-date picture on this disease. I believe this book provides

such a picture. It is intended for scientists and graduate students working in the fields of bacteriology, plant pathology, epidemiology, disease control, plant breeding and molecular plant–microbe interactions. It is not only a reference book on the fire blight disease, its causal agent and methods of control, but I also hope it will provide a springboard for future studies on these subjects. I wish this book will give new hopes for an old dream: understanding how and why *E. amylovora* causes fire blight and how to control this disease.

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Finally special thanks to my family for their support and understanding when I needed to spend so many evenings and weekends back in the office.

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What is Fire Blight? Who is *Erwinia amylovora*? How to Control It?

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Fire blight: a unique disease

Growers and scientists alike would agree that fire blight is like no other plant disease. For growers fire blight is this capricious bacterial disease which can decimate apple and pear orchards in a single season, thus limiting areas where most susceptible apple and pear varieties can be grown (van der Zwet and Bonn, Chapter 3). The economic impact of fire blight is difficult to determine, as losses are not recorded when they are low (a few flower clusters on a few trees killed within a season) and a single severe outbreak can disrupt orchard production for several years. However, over the last few years, fire blight has caused serious losses around the world. In 1998 alone, losses for the north-west USA were estimated to be in excess of US\$68 million (Bonn, 1999), and those for the Hawke's Bay region of New Zealand were estimated to be at least NZ\$10 million. Fire blight also caused severe outbreaks in Lebanon and Emilia-Romagna in Italy, where the previous year 500,000 fruit trees were destroyed due to fire blight (Calzolari *et al.*, 1999). Furthermore, the economic importance of this disease is likely to increase, for several reasons. Firstly fire blight is still spreading geographically into new apple- and pear-growing areas. In Europe, for example, it is spreading west to east and around the Mediterranean Sea and, in 1997, fire blight was detected for the first time in Australia in the Royal Botanical Gardens of Melbourne (Rodoni *et al.*, 1999). Secondly, except for streptomycin, there is still no registered product that can effectively control fire blight (Psallidas and Tsiantos, Chapter 11). Furthermore, the development of streptomycin-resistant strains of *Erwinia amylovora* (Jones and Schnabel, Chapter 12) is threatening the future use of this antibiotic in countries where its use is still permitted. Finally, the new production methods, such as high-density planting, the use of

rootstocks, such as M.26 and M.9, and of new cultivars, such as 'Gala' and 'Braeburn', which are susceptible to fire blight, will also have an impact on the future economic importance of fire blight.

So far, mountains, such as the European Alps, and oceans, such as the Pacific or Atlantic, have not been able to keep fire blight at bay. If it continues to expand geographically as it has done over the last 100 years, it seems inevitable that fire blight will sooner or later reach Asia. Interestingly, apple tree grow wild in some parts of Asia and it is believed that the domestic apple tree actually originated in Central Asia, probably around Kazakhstan and Kirghizstan. If fire blight were to spread into this part of the world, it could lead to the loss of numerous genotypes that have never been in contact with the pathogen before, or it could lead to the discovery of resistance genes to *E. amylovora* among wild apple trees.

To limit the incidence of fire blight, some integrated orchard and nursery management practices have been developed that can significantly reduce the risk of severe outbreaks (Steiner, Chapter 17). These practices rely on orchard sanitation and the timing of chemical sprays to coincide with potential infection events during the blooming period. Such timing is made possible thanks to the development of several risk assessment models (Billing, Chapter 15). Today only heavy metals and antibiotic-type compounds are registered for control of fire blight; the best antibiotic, streptomycin, is not registered in every country where fire blight occurs (Psallidas and Tsiantos, Chapter 11) and, as pointed out earlier, development of streptomycin-resistant strains jeopardizes its effectiveness (Jones and Schnabel, Chapter 12). In the future, novel compounds that do not directly affect the pathogen but interfere with the physiology of the plant, either regulating plant growth or inducing plant defence reactions, such as prohexadione-calcium (ApogeeTM, BASF), harpin (MessengerTM, Eden Bioscience) or BHT (1,2,3-benzothiadiazole-7-carbothioic acid *S*-methyl ester) (sold as BionTM or ActigardTM by Novartis), might be registered for control of fire blight (Steiner, Chapter 17). In addition to these chemicals, a strain of *Pseudomonas fluorescens* (A506) has recently been commercialized in the USA under the name BlightBan A506TM (Plant Health Technologies) as a biocontrol agent for fire blight (Johnson and Stockwell, Chapter 16). Other bacterial strains that could also be used as biocontrol agents are being considered for commercialization, such as *Erwinia herbicola* C9-1 in the USA and *E. herbicola* P10C in New Zealand. These biological control agents might offer an alternative to chemical treatments while waiting for the development of cultivars and rootstocks of apple and pear bred for their natural resistance to fire blight (Lepinasse and Aldwinckle, Chapter 13) or being engineered using genes that code for antibacterial compounds (Norelli and Aldwinckle, Chapter 14).

For scientists, several characteristics set fire blight apart from other bacterial diseases. The development of fire blight symptoms, for example, allows us to distinguish this disease from others. *E. amylovora*, in contrast to most plant-pathogenic bacteria that induce necrosis, can travel rapidly and extensively from the point of infection (Vanneste and Eden-Green, Chapter 5). On susceptible cultivars, if conditions (including climate and the physiology of the tree) are

favourable, the disease can migrate from one infected flower down to the rootstock, killing the tree in a season. Fire blight is an evolutive, necrogenic disease. This ability to migrate long distances through the cortical parenchyma, without producing any enzyme that would help to dissolve the tissues, is quite remarkable. Also remarkable is this ability of the pathogen to spread and to survive within host tissues (Vanneste and Eden-Green, Chapter 5), while unable to survive as an epiphyte (Thomson, Chapter 2). The overwintering in sometimes inconspicuous cankers and the existence of symptomless carriers are two very significant stages of the pathogen's life cycle, which can probably explain some of the sudden outbreaks of fire blight.

The limited host range of *E. amylovora*, with no recognized pathovars, is also worthy of interest. The host range is limited to some plant species that belong to the *Rosaceae*, with the majority of them belonging to the subfamily called *Maloideae* or *Pomoideae* (Momol and Aldwinckle, Chapter 4). These plants, grouped together on the basis of their flower morphology, must have in common a factor that makes them susceptible to fire blight. However, to date there is no indication about the nature and the function of this putative factor, or whether there is only one factor or a family of functionally similar factors.

Finally, fire blight also holds a special place for those interested in the history of science. Indeed, it is the first disease shown to be caused by a bacterium, and *E. amylovora* is the first plant pathogenic bacterium shown to be spread by insects (Baker, 1971).

***E. amylovora*: a unique pathogen**

E. amylovora is the only bacterium capable of inducing fire blight. It is a Gram-negative bacterium belonging to the *Enterobacteriaceae* and its anatomy, physiology and serology have been well described (Paulin, Chapter 6). Microbiologists can, with a few tests, easily separate isolates of *E. amylovora* from those belonging to other species. However, what distinguishes *E. amylovora* from other *Enterobacteriaceae* is a series of relatively minor differences. It is, for example, able to grow, although weakly, under anaerobic conditions and it is unable to reduce nitrate to nitrite. Until recently, one of the most amazing characteristics of *E. amylovora* was the great homogeneity of this species (Vanneste, 1995). However, with the advent of new molecular tools that allow detection of the most minute differences in a bacterial genome and with the finding of isolates that can induce symptoms on selected host ranges (Momol and Aldwinckle, Chapter 4), *E. amylovora* does not look like the monolithic species it once was thought to be. It is still, however, quite a homogeneous species.

In the 1980s, the development of molecular biology techniques enabled the identification of factors involved in its pathogenicity (Vanneste, 1995). Mutants that had lost their ability to induce disease were screened from thousands of transposon-induced mutants, which led to the identification of three groups of genes. Genes called *ams* involved in the biosynthesis of amylovoran, the major

exopolysaccharide produced by *E. amylovora*, those necessary for pathogenicity and the induction of a hypersensitive reaction in non-host plants, called *hrp* genes, and those involved solely in disease development and called *dsp*, standing for disease-specific. Amylovoran is a complex exopolysaccharide made up of a large number of repeating units (Geider, Chapter 7). The sugar composition and the linkages between the sugar residues make this unit unique. However, it is quite similar to the units constituting stewartan, the exopolysaccharide produced by *Erwinia stewartii*, and to those constituting the exopolysaccharide of *Erwinia pyrifoliae*. *E. stewartii* and *E. pyrifoliae* are both closely related to *E. amylovora*. The small differences in the structure of amylovoran and stewartan are actually functionally important. A mutant of *E. amylovora* deficient in amylovoran production could not be complemented for pathogenicity on apple seedlings by the genes necessary for stewartan production. However, production of a specific exopolysaccharide is not sufficient in itself to account for all pathogenicity characteristics of *E. amylovora*, such as host range. A mutant of *E. stewartii* not producing stewartan can be complemented for virulence on maize with genes coding for amylovoran, but such a strain is still not virulent on pears (Bernhard *et al.*, 1996). This confirms that amylovoran is necessary but not sufficient for the development of fire blight symptoms.

The *hrp* genes are necessary for the regulation, secretion and production of proteins – in particular, those called harpins – which seem to interact with the plant cell wall and are necessary for pathogenicity in host plants and the induction of a hypersensitive reaction on non-host plants (Kim and Beer, Chapter 8). A large number of *hrp* genes code for a specific secretion system, called the type III secretion pathway. This pathway is not specific to *E. amylovora* or to plant-pathogenic bacteria; it is shared with animal and human pathogens, such as *Yersinia* spp. In itself this pathway cannot account for the specificity of the symptoms induced by *E. amylovora*. However, it is tempting to hypothesize that the proteins or factors secreted through this pathway are either responsible for or confer the pathogenicity specificity of *E. amylovora*. Two different types of factors secreted via the type III apparatus have so far been identified: harpins and avirulence factors (Kim and Beer, Chapter 8). The harpins coded by *hrpN* and *hrpW* in *E. amylovora* are functionally similar to harpins from other plant-pathogenic bacteria. They are able to induce some rapid changes in the plant cell metabolism, which lead to plant cell death. The avirulence factors do the same, providing the host plant carries a specific complementary resistance gene. Several genes coding for avirulence factors have been identified in *E. amylovora* (Bogdanove *et al.*, Chapter 9). Of particular interest are the genes *dspEE*, which are similar to avirulence genes found in other plant-pathogenic bacteria and which function as such when introduced into those bacterial species (Bogdanove *et al.*, Chapter 9). More genes whose product is secreted via the type III pathway might be identified and some of them might be specific to *E. amylovora*. However, it is significant that so far no factors that can explain the specificity of *E. amylovora* as a plant pathogen have been identified. Could it be that the combination of factors involved in virulence, rather than one or a few

factors in particular, is responsible for the ability of *E. amylovora* to cause fire blight?

In addition to harpins, avirulence factors and exopolysaccharide, only one other factor involved in virulence has been identified. It is the production of desferrioxamine E, a siderophore, which is necessary for disease development when *E. amylovora* is sprayed on flowers (Expert *et al.*, Chapter 10). The role of siderophores in virulence has been well established in the case of animal pathogens and some plant pathogens. It is quite exciting to see, yet again, another parallel between virulence mechanisms in animal pathogens and plant pathogens.

In 1995, Vanneste wrote: 'we have yet to find what makes *E. amylovora* the fire blight pathogen'. Five years later, although we know much more about the disease and the intimate relationship between *E. amylovora* and host plants, we still do not know what makes *E. amylovora* the fire blight pathogen. Trying to answer this question and how to control fire blight better are clearly the next challenges in fire blight research.

Conclusion/summary

Fire blight as a disease and its causal agent *E. amylovora* as a plant-pathogenic bacterium are both unique. After more than a century of studies and thousands of publications, we know a great deal about both the disease and the pathogen. Yet we still do not know why only *E. amylovora* causes fire blight, and why fire blight concerns only some plant species that belong to the *Rosaceae*. However, some strategies of control, involving chemicals, biological control agents, cultivars selected or engineered for their resistance, risk assessment systems and orchard and nursery management practices, are being developed and improved. They have allowed and will continue to allow commercial production of apple and pear in areas where fire blight is present.

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The Disease

I

Epidemiology of Fire Blight

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'There is probably no disease of fruit trees so thoroughly destructive as ... fire blight. No disease has so completely baffled all attempts to find a satisfactory remedy ... notwithstanding the great progress made within the last ten years.' This quote by M.B. Waite (1895) reflected the general feelings about the understanding of the disease and the control of fire blight in 1895. It is interesting that the statement is still appropriate over 100 years later. Great progress has been made in the knowledge of the epidemiology of fire blight in the last 30 years but, despite the advances, fire blight still causes major losses of fruit and trees and creates economic losses amounting to millions of dollars. Fire blight can strike fear in the heart of any pear or apple grower, not only in countries where fire blight exists but also in countries where it has not yet been found. Fire blight will continue to cause epidemics in the near future because there is no single management strategy to combat the disease and very few new control approaches are being developed. The purpose of this chapter is to review and understand the epidemiology of fire blight disease and determine how this knowledge can be used to improve the control of this devastating disease.

Fire blight apparently evolved on indigenous American plants, such as hawthorn (*Crataegus*), native crab apples (*Malus*), mountain ash (*Sorbus*) and perhaps other rosaceous plants. There may even have been an advantage for trees to be infected by *Erwinia amylovora* (Burrill) Winslow *et al.*, because the occasional blighted spur or branch would accomplish the same purpose as pruning or thinning of fruit, resulting in the invigoration of the tree and renewed fruit-producing wood. Thus trees that were slightly susceptible to fire blight would produce more fruit and have a reproductive advantage.

Despite numerous studies and hundreds of publications on the epidemiology of fire blight, there is a constant emergence of new information about this

organism. It is rather comforting that much of the early work was fairly accurate in elucidating the details of a complex disease cycle, despite the limited techniques that were available. Early studies confirmed the presence of the organism by injecting extracts of infected plant materials or insects into young fruit or shoot tips. Today we can readily determine the presence of the organism (Paulin, Chapter 6) by using selective and differential media, such as MSS (Miller and Schroth, 1972) and CCT (Ishimaru and Klos, 1984), PCR (Bereswill *et al.*, 1992), DNA probes (Hale and Clark, 1990) and ELISA or immunofluorescent techniques (Gugerli and Gouk, 1992; Gorris *et al.*, 1996).

The sensitivity of PCR and ELISA reveals the presence of very low populations of *E. amylovora* on or in tissues. The bacteria may be present but at concentrations that have no epidemiological significance. This should be considered when evaluating the potential importance of the detection of bacteria in low numbers.

It appears that *E. amylovora* has the ability to be spread in many diverse ways, explaining why it is difficult to understand and control. The disease cycle will be examined, beginning in the spring, with the sources and modes of dissemination of primary and secondary inoculum in orchards and the processes of shoot infection and systemic movement of the bacteria. The importance of these principles of epidemiology will be related to control strategies.

Sources of primary inoculum

The most probable origin of inoculum to start the spring cycle of fire blight is the spread of bacteria from overwintering cankers to open flowers (Fig. 2.1). Other sources, such as beehives and endophytic and epiphytic bacteria, have been suggested but none have been proven to be the origin of inoculum for new infections.

Cankers and ooze

It is well documented that previous year's cankers are the most likely source of overwintering inoculum (Brooks, 1926; Miller, 1929; Rosen, 1929, 1933; Pierstorff, 1931; Beer and Norelli, 1977), a fact that is easily confirmed when bacterial ooze is present on the surface of cankers. This ooze, composed of viable bacteria in a hygroscopic polysaccharide matrix, may appear as a very sticky and viscous liquid or it may dry to a hard, shiny, amber-coloured glaze. Under low relative humidity the bacteria can survive in the dry exudate for over a year (Rosen, 1938). Thomas and Ark (1934) showed that ants and flies transmitted the bacteria from the bacterial ooze on pear-blight cankers to flowers. Honeybees do not visit the ooze and are not responsible for primary dispersal of inoculum. Only about 10% of the overwintering cankers have viable bacteria, which are usually present in the margins of the canker (Brooks, 1926; Rosen, 1929; Pierstorff, 1931; van der Zwet, 1969). The actual number of cankers that

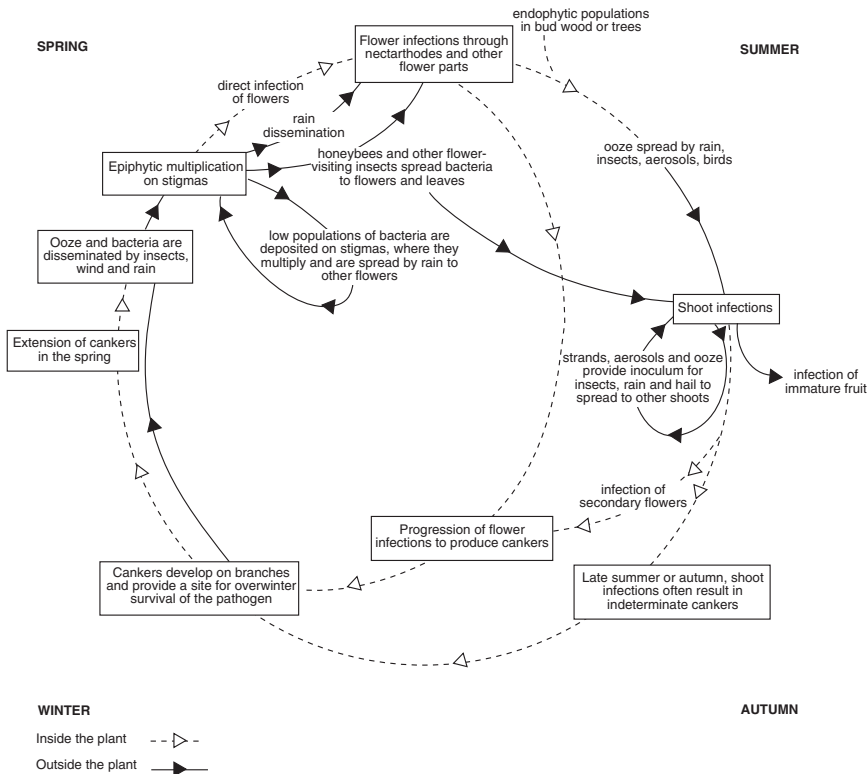


Fig. 2.1. Disease cycle.

ooze and especially those that ooze during bloom is often much less than the number of cankers with viable bacteria. Many cankers are never found oozing, especially those on small twigs, but bacteria were often present in cankers that formed in twigs as small as 0.4 cm in diameter (Brooks, 1926; Ritchie and Klos, 1975). Blighted apple twigs served as important sources of inoculum in Wisconsin during 1926–1928, where there was a clear correlation between the presence of blighted twigs and blossom blight (Miller, 1929). The size of blighted twigs where *E. amylovora* overwinters varied considerably, with some twigs as small as 2–5 mm in diameter. However, the majority of twigs that served as overwintering sites averaged 6 mm in diameter (Brooks, 1926; Miller, 1929). Dye (1949) reported that *E. amylovora* remained viable on fruit spurs following blossom infection until bud movement the following spring.

In many seasons the ooze was present either long before or well after bloom and did not seem to serve as a primary source of inoculum for flower infection. The presence of visible ooze on overwintering cankers is not necessary for bacteria to be available. Several studies have shown that large numbers of bacteria

can be isolated from the surface of non-oozing cankers (Miller and Schroth, 1972; Beer and Norelli, 1977). Rosen (1933) reported that fire blight occurred in Arkansas every year during an 8-year period, even though ooze was not found on a single canker in over 3000 acres of susceptible apple cultivars.

Cankers with indeterminant margins (no sharp border between healthy and diseased tissue) are more likely to harbour the bacteria and produce ooze than those cankers with determinate margins (Beer and Norelli, 1977). Indeterminate cankers develop frequently when the infections take place late in the season or on young plants or on certain cultivars.

There is a broad consensus that pruning out overwintering cankers in the early spring reduces the amount of inoculum in the orchard and is an essential component of a fire blight control programme (Brooks, 1926; Miller, 1929; Rosen, 1929; Tullis, 1929). Fulton (1911) showed that 90% of the cankers did not contain viable bacteria 7 days after they were placed on the ground. The number of cankers necessary for an outbreak may be as few as 1–4 ha⁻¹ (Brooks, 1926; Tullis, 1929). Sanitation is very important but it may be insufficient to prevent blossom-blight epidemics because it is nearly impossible to locate and remove every overwintering canker. Populations of *E. amylovora* also have the ability to multiply very rapidly to extremely high numbers in an epiphytic phase on some floral parts (Johnson and Stockwell, 1998).

Rain and dissemination

Rain easily splashes the bacteria from ooze on cankers to flowers and leaves and is responsible for dispersal of the pathogen in geographical areas where rain is common during the bloom period. Even when ooze is not present the bacteria on the surface of cankers would be easily splash-dispersed. Miller showed convincingly over a 3-year study that oozing cankers in the tops of trees were responsible for a cone of downward spread to lower portions of the tree during rain (Miller, 1929).

***E. amylovora* as an endophyte**

Bacteria that multiply in internal tissues without causing disease are normally considered to be endophytic. However, it may be difficult to distinguish between bacteria that are only surviving in tissues and those that are actually dividing. Whether endophytic bacteria serve as inoculum is still unknown. Bacteria found on the margins of cankers are not considered endophytic but are merely surviving. As early as 1933, Rosen postulated that primary inoculum could originate from internal sources; live bacteria in overwintered, apparently healthy buds or extensions of blight from infections the previous year (Rosen, 1933). Baldwin and Goodman (1963) provided data to support the presence of resident or endophytic *E. amylovora* in 40% of healthy apple buds in Missouri.

The identification of isolates was made using bacteriophage isolates from the soil. The authors may have overestimated the presence of *E. amylovora* in the buds since, although phages are sometimes specific, they are also known to infect other bacterial species (Paulin, Chapter 6). However, confirmation of the presence of endophytic *E. amylovora* in healthy buds, obtained from trees with fire blight in areas where fire blight is prevalent, has been documented by several additional studies (Keil and van der Zwet, 1972a; Eden-Green and Knee, 1974; Dueck and Morand, 1975; Bonn, 1979). In a controlled greenhouse study on apple and pear trees, it was shown that *E. amylovora* could survive for up to 2 years in healthy buds and tissues adjacent to old cankers. The isolated bacteria were still virulent on Bartlett pear shoots in the greenhouse (Keil and van der Zwet, 1972a). All attempts to reactivate endophytic bacteria to cause fire blight have failed (Rosen, 1933; Eden-Green, 1972). Although the bacteria have been detected repeatedly inside apple and pear trees over many years, there is no evidence that these endophytic bacteria are responsible for any outbreaks of infection (Shaw, 1934; Keil and van der Zwet, 1972a). The infection events originating from endophytic bacteria may be infrequent and not responsible for major epidemics of fire blight. However, it is often difficult or impossible to determine the origin of inoculum in fire blight outbreaks.

There is speculation that *E. amylovora* resides permanently inside trees that have been previously infected and that some external influence somehow activates the pathogen. An attempt was made to determine if *E. amylovora* resides in the large scaffold limbs or if new infections reinfect trees each season (Stanley, 1997). All apparent infections were removed from 'Rome Beauty' apple trees that had suffered severe blight for 10 previous years. The trees were enclosed in an 'arborsphere' to prevent any contact with external sources of inoculum. The trees inside the 'arborsphere' were not infected with fire blight, whereas the trees outside were heavily infected as usual. This study suggests that *E. amylovora* does not survive inside older trees and that infections originate from external sources each year. However this contradicts many other studies showing that the pathogen is often detected inside healthy tissues and may be endophytic (Vanneste and Eden-Green, Chapter 5). The age of the trees or the cultivar infected might also be important.

Endophytic inoculum in buds and propagative materials

Several studies have shown that *E. amylovora* is present in a low percentage of buds used for the propagation of trees. Van der Zwet (1983) showed that only 0.5% of pear trees became infected with fire blight when budded with scion wood collected from blight-infected trees. Another study showed that *E. amylovora* could be isolated from 60% of the apparently healthy suckers that developed from blighted 'Bartlett' pear trees in the orchard (Keil and van der Zwet, 1972a). These same researchers have never been able to induce fire blight symptoms in trees containing internal resident bacteria. McManus and Jones (1995) detected *E. amylovora*, using PCR, in an alarming 73% of the axillary

buds taken from shoots in a scion orchard in Michigan. The samples were taken at the same time that workers were collecting bud sticks for grafting and there was no fire blight visible at the time of collection. The significance of these findings is uncertain, since the PCR technique does not distinguish between live and dead cells. It is possible that there was no live *E. amylovora* present, since it could not be isolated. However, these results clearly indicate the possibility for *E. amylovora* to be present in buds or trees and to be unknowingly transported into previously clean areas.

Endophytic bacteria in new trees and nurseries

Primary outbreaks of fire blight in newly planted orchards occur occasionally in the first year of growth, on trees both with and without flowers. In some cases, as many as 10% of the trees are infected with fire blight, usually without any pattern or indication of origin (S.V. Thomson, unpublished results). These random infections are not associated with overwintering cankers on any of the trees. It is possible that the origin of inoculum is from overwintered cankers in nearby orchards, but in many cases the infections occur where the closest known fire blight hosts are long distances away. There is speculation and suspicion by growers and consultants that the pathogen was present in the nursery trees and was not expressed until something triggered the infections (van der Zwet and Walter, 1996). Calzolari *et al.* (1982) isolated *E. amylovora* from vegetative buds of 'Jonagold' apple imported into Italy from a Dutch nursery. The evidence suggests there is potential for fire blight to be transported long distances in nursery trees.

Ornamental hosts

Primary and secondary inoculum can also originate from ornamental hosts either intentionally planted or growing wild in the vicinity of orchards, and this constitutes another frequently ignored and major source of inoculum. The host range of fire blight is quite broad and includes *Crataegus* (hawthorn), *Cotoneaster*, *Pyracantha*, *Malus* (crab-apples), *Photinia* and others species that grow wild or are used in landscape plantings (Momol and Aldwinckle, Chapter 4). Fire blight is often not obvious on some cultivars of these hosts and may be totally overlooked even by someone familiar with the disease (Billing *et al.*, 1974). The symptoms often do not include necrosis of leaves or shoots – only a few flowers or an inflorescence may be infected. Therefore, it is difficult to detect the infection unless very close examinations or isolations are made (Fig. 2.2; see Plates 13a and b). Ooze is produced on infected flowers and many insects visit flowers of these ornamental hosts in huge numbers, contributing to fire blight epiphytotics in northern Europe (Billing, 1978). *Crataegus* is often implicated because it is commonly planted as hedgerows as well as ornamental trees, but *Pyracantha*, *Cotoneaster* and crab-apples are also prime candidates as inoculum



Fig. 2.2. *Pyracantha* plant heavily infected with fire blight on nearly every flower cluster (a), but necrosis and ooze are only evident upon close examination of the flower pedicels (b). The brown fruit are infected with fire blight and will not develop. The infections often stop at the junction of flowers. Foliage and twigs are usually not infected.

sources (Billing, 1980; Schouten, 1992; Paulin *et al.*, 1993). The bloom period of these hosts may not overlap with pear or apple but flowers may not be the only source of inoculum. The small infections in twigs and spurs may be a

major source of inoculum in early spring and may also be a source of inoculum for shoot blight later in the year.

Bees and survival in hives

There is no evidence in the literature indicating that *E. amylovora* overwinters in hives and that bees may be an important source of primary inoculum (Gossard, 1916; Gossard and Walton, 1922; Pierstorff and Lamb, 1934; Hildebrand and Phillips, 1936). Bacteria may survive in hives for several weeks but it is unlikely that they are returned to flowers after natural deposition in the hive (Pierstorff and Lamb, 1934; De Wael *et al.*, 1990). There has been considerable research on the role of honeybees in fire blight epidemiology, but bees are only important in secondary spread of the pathogen from colonized flowers to newly opened flowers.

Soil and survival

Studies conducted in 1932, prior to the availability of good selective media or identification techniques, suggested that *E. amylovora* may survive in non-sterile soil for a few weeks but long-term survival is not considered likely (Ark, 1932). It is interesting that bacteriophages that lyse *E. amylovora* are readily isolated from soil beneath apples and pears (Baldwin and Goodman, 1963; Hendry *et al.*, 1967; Vanneste and Paulin, 1990), which suggests the presence of *E. amylovora* in the soil. However bacteriophages are not specific to *E. amylovora* and often have a host range that includes several species (Hendry *et al.*, 1967; Vanneste and Paulin, 1990). Soil cannot be totally overlooked as a source of inoculum, but it seems unlikely that *E. amylovora* would be splashed from soil into flowers or on to shoot tips. This type of dispersal would be more likely in tree nurseries, where foliage is close to the soil.

Fruit

Fruit infected with fire blight often shrivels and remains attached to the tree through the winter. There are several reports that *E. amylovora* overwinters in these fruit mummies (Anderson, 1952; Goodman, 1954), although the importance of this method of survival is not understood, since there are no documented cases of mummies serving as sources of primary inoculum. Most fruits decompose quite rapidly on the soil and those that remain hanging in the tree usually do not show any evidence of oozing or release of bacteria in the spring.

Immature fruit in orchards with high levels of flower colonization by *E. amylovora* and fire blight infection have populations of *E. amylovora* on the dry flower parts in the blossom end of fruits. However, the bacteria were not present

at harvest (Hale *et al.*, 1987). This phenomenon occurs because *E. amylovora* grows epiphytically on the stigmas in the developing flowers and survives in this protected location (Thomson, 1986; Hale *et al.*, 1987) whereas bacteria on the surface of the fruit die within a short time (Anderson, 1952).

Populations of *E. amylovora* are rare on mature fruit and when present are probably due to deposition from a nearby source of active fire blight (Dueck, 1974; Dueck and Morand, 1975; Hale *et al.*, 1987; Roberts *et al.*, 1989). In every case where *E. amylovora* has been detected on fruit, it has been from orchards with high levels of fire blight infection. Van der Zwet *et al.* (1990) recovered *E. amylovora* from inside mature apple fruit only when it was grown within 60 cm of visible fire blight infections. The recovery of *E. amylovora* from mature apples in British Columbia was undoubtedly also due to the presence of epidemic fire blight on the interplanted pear trees (Scholberg *et al.*, 1988). Despite these reports, it has never been demonstrated that mature fruit are involved in dissemination of *E. amylovora* and serve as a source of new infections in orchards. It would be extremely unlikely that contaminated fruit could be responsible for establishing new outbreaks of fire blight (Roberts *et al.*, 1989; van der Zwet *et al.*, 1990; Thomson, 1992b; Hale *et al.*, 1996).

Movement by animals

Birds have been implicated (Meijneke, 1974; Seidel *et al.*, 1994) for long-distance spread in Europe but there is only circumstantial evidence for such transport. Bech-Andersen (1974) demonstrated the feasibility of bird transport in Denmark. Viable *E. amylovora* were isolated from starling excrement and from their feet 8 days after they were artificially infested with the bacteria. He noted that starlings (*Sturnus vulgaris*) and warblers (*Phylloscopus trochilus*) commonly feed and find shelter in hawthorn hedges, and they complete their migration from England to Denmark in 2–3 days (van der Zwet and Keil, 1979).

Human-assisted spread of *E. amylovora* can occur through infested bud wood (Bonn, 1979; van der Zwet and Walter, 1996), infested nursery stock (Calzolari *et al.*, 1982) or pruning tools (Stewart, 1913; van der Zwet and Keil, 1979; Teviotdale *et al.*, 1991). The technique of girdling can result in significant spread of fire blight (Gardner, 1927), although *E. amylovora* is not spread on pruning tools during the dormant stage of the tree (Lecomte, 1990).

Sources of secondary inoculum

Whether by rain or insects, the arrival of the pathogen on flowers allows for rapid multiplication and dispersal to other flowers. It appears that insects and rain both play an important role in secondary dissemination, depending on the environment. The concern about which is more important seems futile and unnecessary, since both are significant in different situations.

Miller and Schroth (1972) developed a selective medium that allowed them to monitor the populations of *E. amylovora* on healthy flowers, leaves, fruit and insects. They showed that *E. amylovora* could be isolated from apparently healthy flowers prior to infections. However, all of their *E. amylovora* detected on leaves, fruit and insects occurred after fire blight was present in the orchards. Thomson *et al.* (1975) clearly showed that pear flowers were often colonized up to 2 weeks prior to infections and the level of colonization of healthy flowers could be used as a guide for timing sprays (Billing, Chapter 15).

Ooze from new infections

When bacteria enter the flower through natural openings or injuries, they begin to multiply in the intercellular spaces (Bachmann, 1913). The first outward expression of symptoms is water soaking, followed soon by small droplets of ooze on the flower pedicels. The infection may progress down the pedicel and into all the flowers of a cluster. The ooze is a viscous, sticky polysaccharide, which is particularly suited for subsequent spread (Eden-Green and Knee, 1974). Ooze is also a diagnostic indication that the infection is caused by *E. amylovora*.

The presence of ooze accompanied by warm temperatures and rain provides ideal conditions for spread and infection by this well-adapted pathogen (Hildebrand, 1939). Secondary spread can be to other flowers but it also spreads to foliage. The timing of secondary spread is often concurrent with a flush of new, highly susceptible shoot growth.

Strands

Under unique environmental conditions, dry strands of bacteria are observed on infected pedicels, fruits and shoots of all hosts. These strands were studied by Ivanoff and Keitt (1937) and considered important in dissemination. The strands are easily broken off the plant surfaces and transported by wind currents (Bauske, 1967, 1971). Since they are composed of the same polysaccharide material as the ooze, they too are readily rehydrated with water (Keil and van der Zwet, 1972b). Once the strands are rehydrated, the bacteria are only viable for a few days (Eden-Green and Billing, 1972). It seems likely that, under some conditions, strands could be an important mechanism of secondary spread. However, their importance is uncertain, since there are no documented studies showing that new infections are due to the spread of strands. They are probably not important in early blossom infections, since they are only produced during infections in the main bloom period.

Rain

Rain splashing is responsible for both primary and secondary spread to additional flowers or foliage. McManus and Jones (1994), using an Anderson spore sampler, collected *E. amylovora* in 100% of the air samples taken during rain near active shoot infections with conspicuous ooze. However, only 30% of the samples taken during dry weather contained *E. amylovora*. We have isolated high populations of *E. amylovora* from 80% of the water droplets collected from blighted trees during a rainstorm (S.V. Thomson, unpublished results). The dried ooze also rehydrates quickly during rain and is splash-dispersed even after only a few minutes of rain (Eden-Green, 1972). Because dry ooze is so readily rehydrated, it is difficult to find the ooze on cankers after a rainstorm, but brown stains on the bark indicate where the ooze was present before the rain.

Dissemination by insects

Newly opened flowers are normally free of bacteria and remain free if protected from insect visitation or rain splash (Thomson *et al.*, 1975; Thomson, 1983; Miller, 1984; Johnson and Stockwell, 1998). However, if conditions are favourable and there are sufficient sources of inoculum, colonization of nearly every flower in an orchard can take place surprisingly fast (Fig. 2.3). The incidence of flowers colonized by *E. amylovora* changed from 0 or 5% to nearly 100% in 2 warm but dry days in a 2.5 ha Jonathan apple orchard with numerous oozing cankers (S.V. Thomson, unpublished data). An indication of the rapidity and efficiency of honeybees at spreading bacteria is indicated in a study where an antibiotic-resistant marked strain of *E. herbicola* was disseminated to 92% of the flowers in a 2.6 ha apple orchard in 48 h (Thomson, 1992a). Although this was not *E. amylovora* and the inoculum originated from a beehive insert, it clearly shows the potential for extremely rapid spread to nearly every flower in the orchard.

Insects are definitely involved in secondary spread, and many reports and lists of potential insects have been published (Burrill, 1915; Stewart and Leonard, 1915, 1916; Gossard and Walton, 1922; Brooks, 1926; Parker, 1936; Emmett and Baker, 1971; Miller and Schroth, 1972). Van der Zwet and Keil (1979) summarized the long list of insects, with honeybees, aphids, pear psylla (*Psylla pyricola* Foerst.), tarnished plant bug (*Lygus pratensis* L.), leafhoppers and numerous flies as the most commonly mentioned. Honeybees have sharp tarsal claws and stiff bristles, which could also cause microscopic injuries while foraging for nectar or pollen, thus allowing the bacteria entry into the tissues (Schroth *et al.*, 1974).

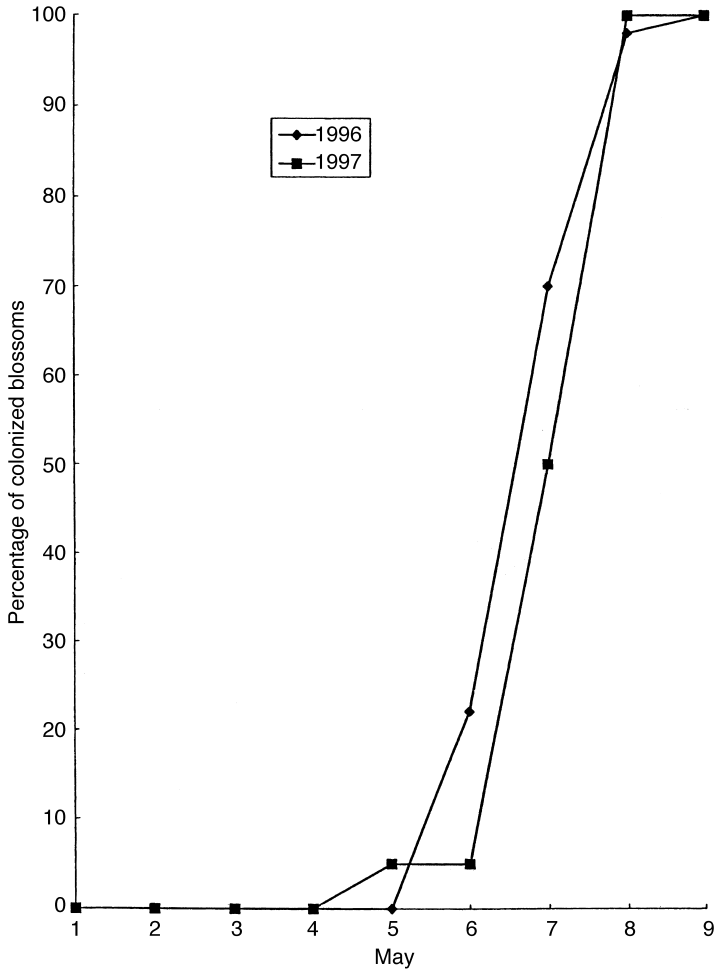


Fig. 2.3. Colonization of 'Jonathan' apple blossoms during a rainless period as detected by stigma imprints in 1996 and 1997 in a 2.5 ha orchard where oozing cankers provided natural inoculum. The incidence of colonized flowers increased from near 0 to 100% in only 2–3 days. Fire blight occurred 10 days after the first detection.

Epiphytic considerations

E. amylovora is not generally considered to be a very good epiphyte and populations usually decline rapidly on most flower parts or leaves within a few hours or days (Miller, 1984). Alternatively, Blakeman (1993) found that *E. amylovora* survived on the surface of cotoneaster leaves for nearly 6 months, but the conditions were artificial, since the study was conducted in a growth cabinet with

the relative humidity maintained at 90%. The exception to limited epiphytic growth under natural conditions seems to be on the stigmatic areas on pistils. Thomson (1986) showed that natural populations of *E. amylovora* occur almost exclusively on pistils, with populations often reaching 10^6 – 10^7 colony-forming units (cfu) per healthy flower. These colonized flowers do not appear any different from normal flowers and usually develop into healthy fruit. This process occurs on all hosts examined, including apple, pear, hawthorn, pyracantha and cotoneaster, and in diverse climates, including California, Utah and New York in the USA, England (Wilson *et al.*, 1989b) and New Zealand (Thomson, 1986; Hale *et al.*, 1996). Hattingh *et al.* (1986) were able to show with scanning electron microscopy that *E. amylovora* and *Erwinia herbicola* multiplied predominantly on the stigmas when apple blossoms were sprayed with suspensions of bacteria. The high populations of bacteria that develop on the stigmas compared with other aerial surfaces indicates that it is a unique bacterial habitat. For example, the epiphytic pathogen *Pseudomonas syringae* pv. *syringae* has been reported to attain sizes of $\sim 10^6$ cfu g⁻¹ fresh weight of bean leaves. However, the populations on the stigma are estimated to be 10^9 – 10^{10} cfu g⁻¹ (Johnson and Stockwell, 1998).

Wilson *et al.* (1989a, b, 1990) examined the growth and development of *E. amylovora* on *Crataegus* stigmas, anthers and nectaries, using isolation of viable bacteria and light and electron microscopy. They found that *E. amylovora* developed in a biphasic manner, with an epiphytic phase up to 48 h after inoculation, where the bacteria were detected only in the intercellular spaces between the papillae on the stigma (Fig. 2.4). At 48–72 h, the bacteria invaded the secretory

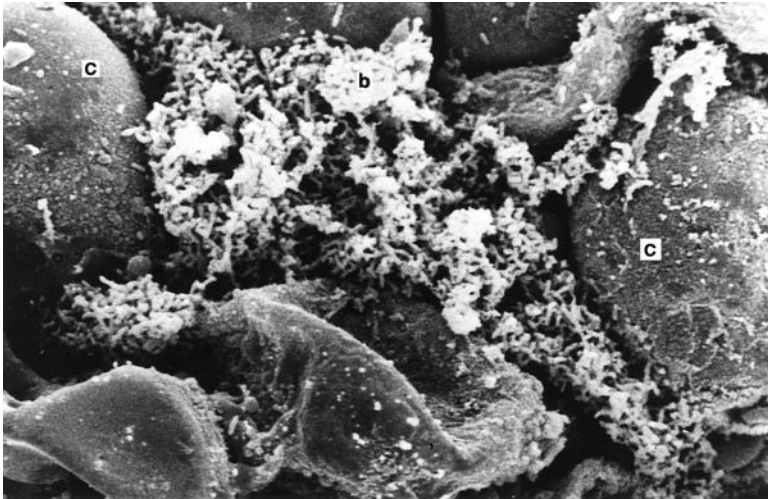


Fig. 2.4. Fire blight bacteria (b) filling the intercellular spaces between the papillae (c) on the stigma at 48 h after inoculation ($\times 1720$). (Original photo courtesy of Mark Wilson.)

tissue below the papillae and formed lysigenous cavities between the collapsed papillae. From 72 h onward, the bacteria completely covered the stigmatic surface and the layer of papillae had collapsed and was necrotic. Despite populations of 10^5 – 10^8 cfu per flower on the pistils, the styles were not invaded and infection did not occur.

Wilson *et al.* (1989a) also showed that a population of 10^8 cfu ml⁻¹ painted on the surface of anthers resulted in growth on the dehiscence zone and junctions of cell walls. The bacteria invaded the anther locule and contaminated the pollen with high populations of *E. amylovora* (Fig. 2.5). Thus contaminated pollen may also serve as a means of disease spread.

The presence of high populations of epiphytic bacteria on healthy flowers provides for efficient movement of the bacteria from flower to flower by rain or by any insects that visit the flowers. Honeybees and flies are particularly effective, providing numerous opportunities for this transfer to take place repeatedly, long before environmental conditions that favour infections occur. In fact, in most cases, the conditions for infection are not met and flowers develop normally into healthy fruit. The normal foraging behaviour of honeybees and other pollen- or nectar-feeding insects is highly conducive to contamination of the stigma by the pathogen (or biological control bacteria) and subsequent spread to other flowers. The abdomen of insects frequently comes in contact with the stigmatic surfaces, both when acquiring the bacteria and later when inadvertently depositing the load of bacteria (Fig. 2.6).

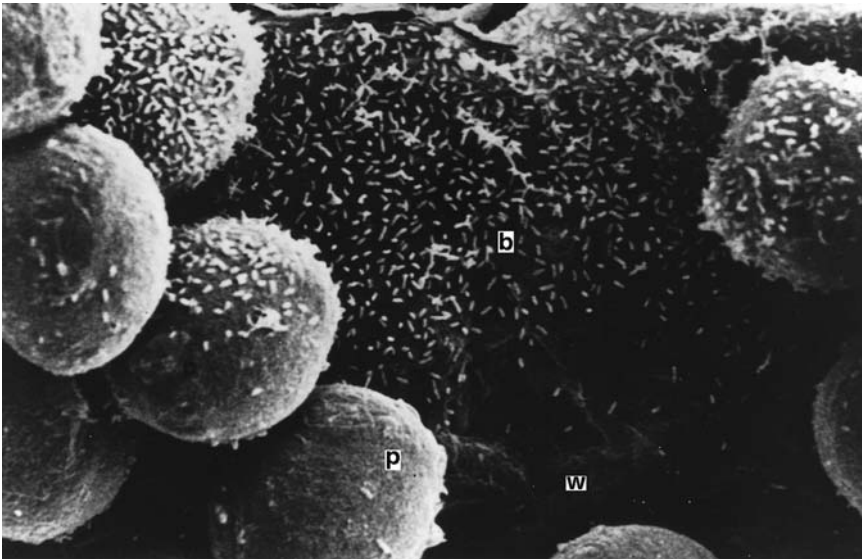


Fig. 2.5. Fire blight bacteria (**b**) on the interior of the locule wall (**w**) and on the pollen grains (**p**) ($\times 1290$). Pollen infested with *Erwinia amylovora* could be a mechanism of dispersal. (Original photo courtesy of Mark Wilson.)



Fig. 2.6. A syrphid fly feeding on a pear flower with contact of the abdomen on the stigmatic surfaces. Some insects often lick the stigmatic surface, while most insects visit the flowers to feed on nectar or pollen.

Sampling techniques and epiphytic populations

The detection of *E. amylovora* populations on flowers has traditionally been accomplished by washing flowers and plating the washings on selective or differential media (Miller and Schroth, 1972; Ishimaru and Klos, 1984; Thomson, 1992c). This process is tedious, expensive and labour-intensive when processing large numbers of flowers. A simple stigma imprint technique can be used to determine the incidence of flower colonization (Thomson, 1992c). Suspect flowers are gently touched and rubbed on an appropriate growth medium so that the stigmas come into contact with the surface of the medium (Fig. 2.7). Since stigmas are the most likely site to be colonized on blossoms, this gives a rapid estimate of the presence or incidence of *E. amylovora* in an orchard.

A comparison of blossom washing and stigma imprints on 'Bartlett' pears during full bloom indicated the sensitivity of the technique. Bacteria were detected on the stigma imprints at lower concentrations than washing and was more sensitive at detecting flower colonization (Table 2.1) (S.V. Thomson, unpublished results). Imprinting the pistils may be more sensitive because populations are not diluted by washing and not mixed with other saprophytic bacteria present on the flower. It may also avoid the release of inhibitory compounds from the plant in the process of washing. Gouk *et al.* (1993) concluded that flower washing and stigma blotting yielded the same information about



Fig. 2.7. The stigma imprint technique showing the contact of the stigmas on the surface of the medium. Contact of other flower parts with the medium is usually irrelevant.

Table 2.1. Comparison of blossom washing and stigma imprints for detecting *Erwinia amylovora* in inoculated ‘Bartlett’ pear flowers. Blossoms were washed in 1 ml of sterile phosphate-buffered saline and 0.1 ml spread on CCT medium. Stigma imprints were also made on CCT. The imprinting technique was more sensitive than washing and even low levels of inoculum resulted in infection.

Inoculum (ml ⁻¹) ^a	3h after inoculation			2 days after inoculation			Infection after 11 days
	Blossom wash		Stigma imprints	Blossom wash		Stigma imprints	
	Log cfu per blossom ^b	Recovery (%)	Recovery (%) ^c	Log cfu per blossom	Recovery (%)	Recovery (%)	
5.0 ^b	3.7 ^b	100	100	3.8	100	89	+
4.0	2.1	50	100	3.5	100	89	+
3.0	0	0	78	3.6	67	50	+
2.0	0	0	22	3.5	33	17	+
1.0	0	0	6	0	0	6	+
0	0	0	0	0	0	0	—

^a Blossoms spray-inoculated at full bloom at noon using a pressurized hand-held bottle.

^b Means are expressed as log₁₀ (colony-forming units) of 18 washed blossoms. Only blossoms with populations were included in the calculations.

^c Percentage of 18 blossoms with *E. amylovora* detected.

the incidence of colonization, but the washing and culturing technique provided quantitative data about flower populations that could not be obtained with the stigma imprint technique. Stigma imprints can be used to detect the presence of biological control bacteria by using the appropriate medium and might be used to monitor orchards to determine when to apply bactericides.

Flower age and susceptibility

Pear flowers were shown to be susceptible for 2 days after opening, but susceptibility declined rapidly with flowers older than 2 days (Hildebrand, 1937). The basis for this decline might be explained by the work of Gouk *et al.* (1996), who showed that *E. amylovora* was unable to grow on the stigmas of 'Royal Gala' apple flowers older than 4–5 days. This may be related to the normal degeneration of the papillae on the stigma of most apple cultivars, which occurs within 2–3 days after flowers open (Braun and Stosser, 1985). Thus pollen germination and fruit set occur only in the first 4 days of flowering. Pollination and subsequent fertilization apparently cause major changes in stigma receptivity. There may also be inhibitory compounds produced on the stigma that prevent colonization by microbes as the flowers age.

Infection pathway

Infection of flowers occurs through numerous natural openings, including stigmas and anthers, stomata on the styles, fruit surfaces and sepals, hydathodes and the specialized stomata, termed nectarthodes, located in the hypanthium (floral cup) (Rosen, 1935; Hildebrand, 1937). The nectarthodes are the most common sites for bacterial invasion in pear and apple flowers, although other flower parts are also invaded (Hildebrand, 1937). Early workers reported that there was no cuticle on the stigmas (Hildebrand and MacDaniels, 1935; Rosen, 1936; Hildebrand, 1937) but Heslop-Harrison (1976) showed that *Malus* stigmas are covered by a thin cuticle that is barely visible in the light microscope. This cuticle ruptures as the stigmatic secretions force the cuticle away from the papillae.

Rain or dew facilitates the movement of *E. amylovora* from the stigmas to the hypanthium or to other flower parts where infection may occasionally occur (Thomson, 1986; Wilson *et al.*, 1989b; Thomson and Gouk, 1992). The importance of this simple phenomenon becomes apparent since low populations on most flower parts usually decline and create no infections (Hildebrand, 1970; Lelliott, 1978; Wilson *et al.*, 1990; Hale *et al.*, 1996). This process explains how *E. amylovora* can be deposited in low numbers on the stigma and yet ultimately result in symptoms much later. Thus the speculation by Schroth *et al.* (1974) that fire blight may be an example of a disease where inoculation in nature is often accomplished by massive rather than low dosages of inoculum is

probably only partly correct. The transfer of low numbers of bacteria to the plant occurs with insects or splashing rain, but the growth on the stigma and subsequent transfer by rain results in inoculation by numbers sufficiently high to cause infection.

Support for the importance of rain in movement of bacteria is apparent when outbreaks of fire blight, are synchronized with rainstorms in an orchard. For example, it is often possible to examine an orchard and find no fire blight, only to return 1 or 2 days later to find new blight symptoms on nearly every flower. The simultaneous outbreak of symptoms in orchards indicates that *E. amylovora* infections have been synchronized by some environmental trigger. Steiner (1990) and Lightner and Steiner (1993) demonstrated excellent relationship with rain, hail, wind and dew as the initiators of new epiphytotics. Their Maryblyt model often accurately predicts to the day when new infections are likely to be detected (Billing, Chapter 15). Infections initiated by sequential colonization of flowers and subsequent growth would result in continuous new infections developing over time rather than infections on specific days.

Shoot infections

E. amylovora is not a strict phylloplane epiphyte, according to the definition by Leben (1965), but has a transient nature on leaf surfaces and is usually only present after blossom infections have occurred in the orchard (Miller and Schroth, 1972; Miller and van Diepen, 1978). Crosse *et al.* (1972) were unable to detect *E. amylovora* on apple leaves prior to infections, but large numbers of *E. amylovora* were found on the leaves shortly after the appearance of fire blight symptoms. The role of these transient bacteria in shoot infections is not clear.

The infection of shoots is always first apparent in the actively growing young leaves on the tip. There are occasional leaf spots on older leaves caused by fire blight, but the typical wilting and ooze production do not develop on fully expanded leaves until after the tip has expressed symptoms (Heald, 1915; Rosen, 1929, 1933; Lewis and Goodman, 1966; Crosse *et al.*, 1972). Injury seems to be important in most infections and outbreaks are often associated with hail, strong wind or thunderstorms (Brooks, 1926; Rosen, 1929; Keil *et al.*, 1966). These environmental events may produce very obvious injuries that allow the entrance of bacteria, but some of the injuries are not readily apparent and may be microscopic. Many studies suggest that injury is necessary for infection and only occasional infections occur through uninjured leaves (Brooks, 1926; Pierstorff, 1931; Rosen, 1933).

Importance of injury

Crosse *et al.* (1972) claimed that leaf injury must be sufficiently extensive to expose veins so as to provide the pathogen direct access into the vascular system

of the leaf. Bacteria were sucked into the xylem vessels and moved via these elements to the shoot axis. The chances for infection rapidly diminished as the time from injury increased. There were few infections at 48 h after injury. These results are very similar to those of Brooks (1926), who noted that leaf infections in the veins of the leaves took place almost exclusively through wounds less than 48 h old.

Heald (1915) may have been the first to observe and document that *E. amylovora* can enter uninjured leaves through water pores (hydathodes) and stomata. Most leaf infections started on the margins of leaves, where the hydathodes were located. After the primary entry, the bacteria entered the veins and eventually spread throughout the shoot. There are several documented reports of the pathogen entering stomata on leaves and lenticels on stems (Miller, 1929; Rosen, 1929; Tullis, 1929). Tullis (1929) observed that infections did not occur on very young leaves where the stomata had not yet developed, but later, as stomata became functional, leaf infection occurred. Lewis and Goodman (1965, 1966) found that *E. amylovora* could enter through glandular trichomes and hydathodes on the upper surface of 'Jonathan' apple leaves, which are unique because they do not have stomata on the upper leaf surface. They claimed that injury was not necessary, but the inoculation entailed 'spreading' the bacteria over the serrated margin of the upper leaf surface of the sixth leaf from the tip. There was no obvious injury caused by the inoculation, but certainly the glandular trichomes would be easily broken by even the most gentle spreading technique. The most striking discovery was that the bacteria were detected in the shoot tips 6–7 h after inoculation, but symptoms did not become apparent until 5–7 days after inoculation (Lewis and Goodman 1965, 1966). There were no symptoms on the intervening leaves. *E. amylovora* has been isolated from chlorotic leaf spots on mature leaves of otherwise healthy shoots and also detected inside the apparently healthy shoots, using PCR, in many cases when *E. amylovora* could not be isolated (S.V. Thomson, unpublished results). These studies suggest that shoot blight may be the result of bacteria entering older leaves on the shoots and migrating to the shoot tip, where the tender leaves express the symptoms of blight (Fig. 2.8; see Plate 18). Thus even transient, low populations of *E. amylovora* on mature leaf surfaces may be sources of infection for shoot blight. It may not be necessary for *E. amylovora* populations to be present on the shoot tips for infection to occur.

Shoot blight does not seem to be a random occurrence of new infections. There are definite waves of infection associated with environmental events. Thus, even though the bacteria may be present on the leaves and perhaps even inside the tissue, the infections still seem to be initiated by an environmental event, such as thunderstorms or hail.

Although *E. amylovora* has been detected on leaves, it appears that it is ephemeral and present only after fire blight is present in proximity (Miller and Schroth, 1972; Miller 1984; Manceau *et al.*, 1990). Multiplication could not be demonstrated on leaf surfaces and *E. amylovora* usually died within a few hours when exposed to solar radiation or high humidity (Maas Geesteranus and de



Fig. 2.8. Shoot infection on apple with typical wilting and ooze on the terminal leaves and a leaf spot caused by *Erwinia amylovora* on leaf number six with all intervening leaves still healthy. Suggests that the bacteria entered leaf number six and moved systemically to the shoot tip, resulting in shoot-tip blight.

Vries, 1984). In contrast to these earlier studies, there is evidence that *E. amylovora* was present on 100, 80 and 75% of leaves, axillary buds or in the blossom end of fruit, respectively, when assayed using nested PCR (McManus and Jones, 1995). In many cases, the detection by nested PCR was from an orchard where there were no symptoms of fire blight and where it was rare the previous year. There is no evidence that the cells were alive or that their presence resulted in fire blight. However, these results may be an indication of the widespread nature of *E. amylovora* in populations too low to be detected by the normal culturing methods and in sites where *E. amylovora* is not normally expected.

Systemic movement

The movement of *E. amylovora* upward and downward in the tree seems to depend on the plant tissue that is infected. However, there is no consensus about the primary pathway for migration of the pathogen in plant tissues. Most studies indicate that there is rapid movement through xylem vessels (Bachmann, 1913; Rosen, 1929; Shaw, 1934; Crosse *et al.*, 1972; Aldwinckle and Preczewski, 1976), whereas other studies have implicated rapid movement in the phloem (Lewis and Goodman, 1965; Gowda and Goodman, 1970). Another group of studies provide evidence for migration in the cortical parenchyma (Bachmann, 1913; Nixon, 1927; Eden-Green and Billing, 1974). It would appear that *E. amylovora* has the ability to migrate in most plant parts and it may be different depending on how the infection enters the tissue. Therefore upward and downward mobility of *E. amylovora* in a tree may be variable, depending on where the bacteria enter and other unknown factors.

The rate of movement or the speed of systemic transport of bacteria has been recorded in excess of 15 cm in 7 h (Lewis and Goodman, 1965). Symptom development is very fast and is reported to be up to 30 mm day⁻¹ (Brooks, 1926; Rosen, 1936).

Growers are uncertain about the advantages of pruning out fire blight as a means of slowing the spread of an epidemic and minimizing losses. Studies have shown that less total blight occurs in an orchard when prompt pruning removes inoculum and prevents systemic spread and loss of entire trees (Covey and Fischer, 1990). Since *E. amylovora* has the ability to move systemically, often with surprising rapidity, it is imperative that pruning be done promptly, and frequently with the removal of lengthy sections of healthy tissue adjacent to the infection. When infected shoots were pruned at the base of visible symptoms, 57% of the corresponding stubs remaining on the trees showed visible progression of fire blight after removal of the infection. However, pruning at the normally recommended 20–25 cm beyond visible symptoms resulted in 21% of the cuts being made through tissues with viable *E. amylovora*, but still 12% of the cut stubs resulted in an extension of symptoms (Clarke *et al.*, 1991).

Graft-union infections

Rootstock or collar blight is an important phase of fire blight, but only when susceptible trees are grown on highly susceptible rootstocks, such as the European dwarfing rootstocks, M.9, M.26 and M.29 (van der Zwet and Beer, 1995). Collar blight can occur on trees where there is little or no evidence of fire blight infection on the top, which creates confusion with other fungal root or collar diseases. These graft-union infections could occur as a result of: (i) infected suckers or water sprouts; (ii) washing of bacteria from higher infections down the trunk and into the crown area; or (iii) internal translocation of *E. amylovora* from other infections in the tree. Gowda and Goodman (1970) reported the presence

of *E. amylovora* in the roots within 2 weeks after shoot-tip inoculation and a distance of 70 cm from the point of inoculation. Momol *et al.* (1998) showed that leaf inoculations of *E. amylovora* could move more than 50 cm in 12 days, and by 22 days it was present in the M.26 rootstocks of 2-year-old 'Empire' apple trees. This internal movement to the rootstock is much more likely when infections occur late in the season (Momol *et al.*, 1998).

Importance of understanding epidemiology in control of fire blight

A thorough understanding of the epidemiology of *E. amylovora* is critical in controlling this devastating disease. It is possible to utilize epidemiological knowledge of the pathogen to find weak spots or areas where the pathogen can be eliminated or reduced. For example, knowing that *E. amylovora* is not normally present systemically throughout mature trees and that thorough pruning of overwintering cankers will eliminate most of the surviving bacteria provides support for the careful pruning of dormant trees. It helps us to understand why the expedient and timely pruning of fire blight during the season is useful but delayed pruning is often a waste of time. We also know that there are times when *E. amylovora* is present endophytically in trees or tissues and that total eradication may not be possible. *E. amylovora* is not always present on leaf surfaces, which is probably why foliar applications of bactericides are usually not effective in controlling shoot blight. Foliar sprays are not likely to correspond with the presence of foliar populations and are probably not present at effective concentrations during an subsequent infection period. The knowledge that *E. amylovora* multiplies preferentially on the stigmatic surfaces assures us that we can monitor stigmas for the presence of *E. amylovora* to anticipate control requirements and fire blight outbreaks. The information we now have on colonization sites and the role of rain should enable us to provide better timing and proper coverage of sprays (Psallidas and Tsiantos, Chapter 11). It also indicates the site where biological control interactions take place and where bactericide residues might have the most direct effect. The important and efficient role of insects in vectoring the pathogen reveals how quickly infestations take place and the need for constant vigilance with regard to insect activity.

It becomes very clear after examining the numerous ways that *E. amylovora* survives and is disseminated that satisfactory control is a formidable task. The control of fire blight cannot be obtained with a single 'silver bullet' strategy but requires the utilization of all available knowledge about the epidemiology of the pathogen. It makes one wonder if we shall still have the same feeling about fire blight control as M.B. Waite did in another 100 years.

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Distribution and Economic Importance of Fire Blight*

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The disease known as fire blight, caused by *Erwinia amylovora* (Burrill) Winslow *et al.*, has been reported from 40 countries around the world (Table 3.1). It has spread from its original site in the Hudson Valley of New York State, USA, across the North American continent and to the Pacific Rim, Europe and the Middle East during the past 218 years (Fig. 3.1). We shall discuss the distribution and the economic impact of this disease in the following two sections, with an added bibliography.

Countries within North America

Since the late 18th century in the USA, fire blight has been continuously observed on cultivated and wild species of members of the *Rosaceae*. The earliest known observations of fire blight were made in the Hudson valley of New York State in 1780 (Denning, 1794). From New York the disease spread south and westward over the Allegheny Mountains into the Ohio and Mississippi River valleys. This movement most likely occurred with the planting of fruit orchards by the early settlers as they moved westward. Coxe (1817) in the early 19th century recognized fire blight as a major disease problem for fruit production. Severe blight epiphytotics were experienced on apple (*Malus* spp.) and pear (*Pyrus* spp.) in the eastern US in the first third of the century. About 1840, fire blight, reached Ohio, Indiana and Illinois. Beecher (1844) reported that one of

* Crop loss figures are US dollar values at the publication date of the referenced articles.

† Retired.

Table 3.1. Countries in which fire blight has been recorded, as shown on the distribution map (Fig. 3.1).

Europe	Eastern Mediterranean
Albania	Armenia
Austria	Cyprus
Belgium	Egypt
Bosnia	Iran
Bulgaria	Israel
Croatia	Jordan
Czech Republic	Lebanon
Denmark	Turkey
France	
Germany	
Great Britain	The Pacific rim
Greece	Japan
Hungary	New Zealand
Ireland	Australia
Italy	
Luxembourg	North America
Macedonia	Bermuda
Netherlands	Canada
Norway	Guatemala
Poland	Mexico
Romania	USA
Serbia	
Spain	
Sweden	
Switzerland	

the most widespread and destructive epidemics occurred in 1844, in which many Midwestern orchards were completely destroyed. During the period of 1876–1880, state and county horticultural meetings addressed the issue of fire blight, as the disease had been a major factor in the destruction of so many orchards in the Midwest.

Fire blight also moved southwards into the southern and Gulf coast states with the expansion of settlements and the planting of fruit orchards. The mild humid climates were important factors that explain the destructive nature of the disease in these regions. The vast expanse of the western plains and the mountains seemed to prevent the expansion of fire blight into the Rocky Mountain and west coast states. However, the disease appeared suddenly in pear orchards near Chico, California, in 1888. Soon after, Pierce (1901, 1902) confirmed the identity of the causal agent and the presence of fire blight in California. In the early part of the 20th century, Fresno and Kings Counties appeared to be the centre of the epiphytotics in this state. Detailed accounts of the early history of fire blight in California have been published (Gardner and

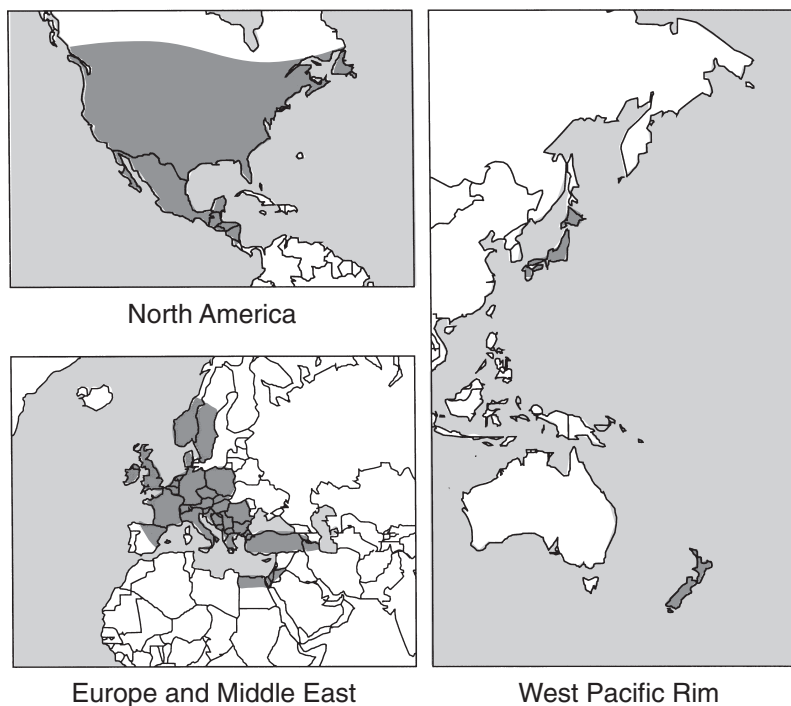


Fig. 3.1. World distribution of fire blight.

Ark, 1924; Baker, 1971). It has been suggested that two-thirds of the pear trees cultivar 'Bartlett' trees were eliminated, at a cost of \$5 million, during the period 1903–1908 (Woods, 1909). Later, Milbraith (1930) estimated fire blight losses at \$2,268,260 for 1930 alone; this estimate includes the cost of crop loss, fire blight control, inspection and autumn fire blight control. Talbert (1925) indicated that young orchards were frequently wiped out, bearing orchards lost 25–50% of their production and the loss of fruiting wood impaired production for several years. Often, it is difficult to assess the economic loss due to fire blight, as there are many factors to consider.

Following its discovery in California, fire blight spread northward into Oregon and Washington, where major epiphytotics occurred later. In Oregon, fire blight appeared in the Rogue River valley in 1908 and somewhat later in the Umpqua and Hood River valleys (O'Gara, 1910). Only after the disease had been reported in parts of Washington State was it observed in the important fruit-growing Willamette valley of Oregon in 1915 (van der Zwet and Keil, 1979). In the Rocky Mountain states, the disease appeared somewhat later than in California, when Swingle (1911) reported its appearance in Montana in 1905.

Since its first discovery in the Hudson valley of New York in 1780, fire blight has moved into every region of the USA. This process took a period of approximately 135 years at a time when the movement of humans and goods advanced from the horse and wagon to rail and car (van der Zwet and Keil, 1979). Without a doubt, human activity has been a very important factor in the spread of fire blight. Host susceptibility and weather have been the important factors in the development of epiphytotics. The economic importance of the disease has been felt throughout the USA over the past three centuries. The early settlers saw, first, the loss of fruit production, particularly pears, which were susceptible European cultivars, such as 'Bartlett' and 'Bosc', and, secondly, the loss of their trees, as they succumbed to the disease (Wendell and Downing, 1848). Pear culture along the eastern seaboard and in the Great Lakes region felt the devastation of the disease, as many orchards fell victim to fire blight throughout the 19th and early part of the 20th century (van der Zwet and Keil, 1979). For this reason, pear culture has been mostly restricted to the drier regions of the west coast states. However, even today, with the introduction of numerous susceptible apple cultivars, fire blight can be a serious threat to pear production in the west.

Production of apple, on the other hand, has not been as seriously affected by fire blight as pear production. Numerous apple cultivars are either resistant or tolerant to fire blight. For this reason, apple production is more common throughout many areas of the USA including the mild and humid regions of the middle and south-eastern states. Only in the last 10 to 15 years have we seen that fire blight can cause serious production problems (Thomas and Jones, 1992). Fire blight was particularly severe in 1991 in south-western Michigan, where the estimate of losses was \$3.8 million (van der Zwet and Beer, 1995). During the 1992–1994 period, the disease was severe in the east, central and western part of the USA. Currently, apple growers are investing heavily in high-intensity plantings of newer cultivars, which are usually highly susceptible (Lespinasse and Aldwinckle, Chapter 13). With the increased level of interest in high-intensity orchards on many sites in North America, there is a real need to assess the damage caused by fire blight through thorough surveys when the problem arises.

The first report of fire blight in Canada appeared in an annual report of the Fruit Growers Association of Ontario (Anon., 1890), in which a grower reported personal observations of fire blight during the previous 25 years. Harrison (1904) speculated that the disease had occurred as early as 1840 and that it had spread to many areas of the province by 1870. This would also coincide with the period when the disease appeared in the neighbouring states of Ohio, Indiana and Illinois (Beecher, 1844). Its economic importance was not noted until an outbreak occurred in the Niagara Peninsula in 1943 (Chamberlain, 1944). For many years afterwards, pear production in the Peninsula was dominated by the 'Kieffer' cultivar, which shows good field resistance to the disease. Fire blight has occurred sporadically in Ontario, with major epiphytotics occurring every 8–15 years. The last survey in 1972 in southern

Ontario revealed that about one-third of the orchards had economic damage due to fire blight (Dueck and Quamme, 1973). More recently, the disease has appeared in nurseries and young plantings of apples with increasing frequency (W.G. Bonn, unpublished observations). Some of this can be attributed to the increasing use of susceptible cultivars and higher tree densities.

Outside Ontario, the disease has been reported in British Columbia, where it was first noted in 1911 (Eastham, 1935); however, in all likelihood the disease was probably present earlier, as it had already appeared to the south in the west coast states of the USA. It was often observed in the interior of the province but that may have been because of the higher intensity of fruit production in the dry interior valleys. More recently, outbreaks of fire blight have been observed in young apple orchards planted with imported nursery stock (P.L. Sholberg, personal communication). Economic losses were sometimes substantial for individual growers.

Fire blight has also been observed in apple plantings in Quebec, even though the climate there is cooler than that of the fruit-growing areas of neighbouring Ontario. Several reports in the 1930s and 1940s (Racicot, 1931, 1938; Thatcher, 1943, 1944) indicated substantial losses for several growers. East of Quebec, in the maritime provinces, fire blight has been reported to have caused losses in New Brunswick and Prince Edward Island in the 1950s and later (Canada Department of Agriculture, 1920–1970). It appears that the disease did not occur much earlier than 1966 in the fruit-growing Annapolis valley of Nova Scotia (Lockhart and Gourley, 1961–1965; Gourley *et al.*, 1967) where some pear loss occurred.

In the harsh climate of the prairie provinces of Manitoba, Saskatchewan and Alberta, fire blight has been reported on cultivated *Rosaceae* (Canada Department of Agriculture, 1920–1970). Nurseries and street-scape plantings have suffered significant losses on cotoneaster (*Cotoneaster* spp.) and mountain ash (*Sorbus* spp.) in some years. It is likely that the disease is endemic in many ornamental plantings in the west. Evans (1996) suggested that fire blight leads to losses in fruit production for raspberries (*Rubus* spp.).

Once again, fire blight is becoming more important in the fruit-growing regions of Ontario and British Columbia, as the growers follow the world trend toward planting high-density orchards of susceptible apple cultivars and susceptible rootstocks. In addition, fruit growers in the Niagara Peninsula of Ontario have replaced much of their 'Kieffer' pear plantings with susceptible cultivars, such as 'Bartlett', 'Bosc' and 'Anjou'.

Fire blight has been reported on the island of Bermuda, a self-governing territory of the UK, situated 900 km east of the USA in the Atlantic Ocean. In 1938, the disease was listed by Waterston (1938) as occurring on loquat (*Eriobotrya japonica*).

In Mexico, Ramirez (1921) included fire blight in a list of plant diseases and pests observed in the federal district (Mexico City) as early as 1921. In 1943, Robles Gutierrez (1943) reported that fire blight was serious on apples and

pears in the Canatlán region. The disease is now present in most of the apple- and pear-growing regions of Mexico (Lopez and Fucikovsky, 1990). Economic loss evaluations are unavailable.

In 1968, Schieber and Sanchez (1968) included fire blight in a preliminary list of plant diseases in Guatemala. They reported finding the disease on the pear cultivars 'Bosc' and 'Bartlett' in several regions. In Guatemala, pear plantings are rather recent and also somewhat isolated at high elevations. It is likely that fire blight was introduced with the original plant material.

Countries outside North America

The Pacific rim

The earliest report of fire blight outside the North American continent came in 1903 from Japan, where Uyeda (1903) observed the disease on apple trees, presumed to have been brought over on nursery stock from America. He identified the causal organism as *Bacillus amylovorus*, which at that time was the accepted name of the fire blight bacterium. Uyeda performed the reinoculation experiments and described the symptoms of the disease as identical to those of fire blight, including the characteristic scorched appearance of affected leaves and the reddish-brown streaks in the vascular system of affected wood. Fire blight was observed on both apple and pear in Akita Prefecture on the island of Honshu and in Ehime Prefecture on Shikoku island. Kazui (1922) reported the disease in Hokkaido as well as on the northern part of Honshu. The disease was apparently quite severe during the late 1920s (Shiraishi, 1930).

In 1955, Okabe and Goto published a list of bacterial pathogens in Japan and the diseases they cause, which also included a bacterial disease of pear caused by an unidentified *Erwinia* species. In 1992, Goto described a disease in a textbook on bacterial diseases as a 'bacterial shoot blight of pear' (BSBP), which occurred on Hokkaido. This disease was apparently first reported on this island in 1936 by Takahashi (Beer *et al.*, 1996). Symptoms were described by Goto (1992) as identical to those of fire blight and the causal organism as nearly identical to *E. amylovora*, except for some specific, but unidentified, properties. In 1976, Tanii *et al.* (1976) identified the causal agent as *E. amylovora*, but in 1983 reported that several isolates presented distinct pathogenicity on certain Asian pear cultivars, and suggested that the bacterium be named *E. amylovora* pv. *pyri* (Tanii, 1983; Momol and Aldwinckle, Chapter 4). Regardless of host tree specificity or bacterial strain involved, the most recent characterization study concluded that BSBP is identical to fire blight (Beer *et al.*, 1996).

The second report of fire blight outside North America came from New Zealand, where Campbell (1920) and Cockayne (1921) reported the initial outbreaks in 1919 on apple, pear, quince (*Cydonia* spp.) and hawthorn (*Crataegus* spp.). The disease was observed around Auckland in the north of North Island, and is believed to have been imported on nursery stock. Waters (1922) was the

first to isolate and identify the causal organism. Eight years after its introduction, fire blight caused severe losses to most of the apple cultivars in the Hawke's Bay area (Adamson, 1929). During the early years, the disease destroyed many hectares of pear trees in a single season, but, within 2 or 3 years, damage was limited to the loss of a few branches and a small proportion of spurs (Cunningham, 1931). Hawthorn, used extensively as wind-break hedges around fruit orchards, was, and still is (J.L. Vanneste, editorial comment), very instrumental in the overwintering and dissemination of the disease (Cockayne, 1921).

Despite quarantine regulations concerning shipments of bees or plant material from infected areas, fire blight reached South Island in 1929 (Reid, 1930). Following several outbreaks of the disease in other new areas, the disease seemed to become less severe over time (Cunningham, 1931). It was detected in the Otago region (southern part of South Island) in 1936 (Dye, 1970). Dye reported an increase in fire blight severity in the early 1960s and that streptomycin resulted in considerably better control than Bordeaux mixture.

In April 1997, fire blight-like symptoms were observed on three different rosaceous plants in the Royal Botanic Garden in Melbourne, Australia (Rodoni *et al.*, 1999). Rodoni *et al.* also reported that subsequent surveys of the garden, as well as pome fruit orchards and fruit tree nurseries throughout Victoria, up to October 1999, have not revealed any sign of the disease. The most recent attempts to isolate *E. amylovora* from stored plant tissues appeared contradictory, as two reports stated that the bacterium could be isolated (Jock *et al.*, 1999; Kim, J.F. *et al.*, 1999), but another stated that fire blight was at the lower levels of detection (Rodoni *et al.*, 1999).

In 1999, a new species of *Erwinia* (*E. pyrifolia*) was reported to cause necrotic branches on Asian pears (*Pyrus* spp.) in South Korea (Rhim *et al.*, 1999). Disease symptomatology, host specificity and bacterial biology (Kim, W.S. *et al.*, 1999) appear extremely close to those described and mentioned above for BSBP in Japan.

Japan, New Zealand, South Korea and possibly Australia are the only four locations in the total Pacific region where fire blight, or a closely related disease, has been observed and reported. The remaining countries with fire blight are all located in Europe and the eastern Mediterranean region, following introduction of the disease in the UK and Egypt, respectively (van der Zwet and Bonn, 1999).

Europe and the eastern Mediterranean

The information provided below indicates that, sometime during the 1950s, the fire blight organism was most probably disseminated, via infested bud wood or trees, to two different locations in an eastern direction from North America: one in north-western Europe and the other in the north-east corner of Africa.

The first outbreaks in the UK were reported in 1958 by Crosse *et al.* (1958) on pear trees near Maidstone, Kent. Immediately, the disease became very severe on the cultivar 'Laxton's Superb', presumably because of its late-blooming characteristic. It has been suggested, but never proved, that the bacterium may have been introduced on contaminated fruit crates, which were recycled in those orchards in Kent where the initial blight symptoms were observed in 1956/57 (Lelliott, 1959). By 1959, the disease was noted on pears, hawthorn and mountain ash (*Sorbus aria*) in the southern suburbs of London, as well as in the borough of Southend-on-Sea, located across the River Thames from Kent, within 35 km of the infested orchards in Kent (Hodgkin and Fletcher, 1965). By 1966, fire blight had spread east to Canterbury and north and westward to Suffolk and Berkshire. That year was considered one of the worst years for fire blight in England (Great Britain Ministry of Agriculture, Fisheries and Food, 1966). New infections were recorded in 5800 'Laxton's Superb' trees, 1600 'Bartlett's' and 2200 pyracantha and cotoneaster plants. Fire blight was not recorded on apple until 1967 (Lelliott, 1968) but, by autumn 1969, the Ministry of Agriculture announced that severe outbreaks had occurred on more than 1700 trees in 45 apple orchards throughout Kent (Great Britain Ministry of Agriculture, Fisheries and Food, 1969).

During the next 30 years, the disease spread throughout England and into Wales, but today it is of minor economic importance to the fruit industry as a whole (E. Billing, personal communication). It appears that most serious losses occurred to certain extremely susceptible pear cultivars, such as 'Laxton's Superb', and to several cultivars of perry pears, for example cultivar 'Butt'. By 1986, fire blight was reported on six different host plants in 55 sites in Ireland (McCracken, 1987).

Fire blight was not reported on the mainland of the European continent until 1966. The initial reports came from two distant locations in the autumn of that year: one from the islands in the south-western part of The Netherlands (Netherlands Plant Protection Service, 1966) and the other from the Baltic coast of Poland (Borecki *et al.*, 1967). Because the first reports of the disease in Denmark (1968) and the northern coasts of former West Germany (1971), Belgium (1972) and France (1972) all appeared within 6 years, it has been suggested that migratory birds may have been instrumental in the dissemination of the bacterium across the English Channel to the western and northern European coastlines (Meijneke, 1972; van der Zwet, 1994).

In August 1966, fire blight became established in The Netherlands in hawthorn in the south-western corner of the country. Many hedges and wind-breaks surrounding fruit orchards were eradicated in order to reduce sources of inoculum (Meijneke, 1972). The disease also became severe on the long-leaf cotoneasters in parks and gardens. During the late 1960s, it appeared as if fire blight had been eradicated (Netherlands Plant Protection Service, 1970), but in 1971–1973 new occurrences were discovered in the provinces of Zeeland and North Holland, primarily on hawthorn along ditches and roads, on dunes and in private gardens (Meijneke, 1972). Since the early 1980s, fire blight has been reported to be very mild during cool spring weather at bloom time; how-

ever, severe blight has been observed after July, especially in the southern growing regions of the country or following severe hailstorms (M. van Teylingen, personal communication). In 1982, a particularly severe year, the combined economic impact of the disease on nurseries and fruit orchards and the total cost of eradication and control were estimated at \$6 million (Netherlands Plant Protection Service, personal communication).

Fire blight was first observed in France and Belgium in 1972 along the coast of the English Channel (EPPO, 1972). In France the disease was again observed in hawthorn hedges and cotoneaster shrubs. By 1978, the disease had spread inland to Lille and, by 1983, further towards the centre of the country (Callu, 1984). In 1984, fire blight was quite severe in the south-west area of the country, in particular on the cultivar 'Passe Crassane' (Lecomte *et al.*, 1984). The following year, it was reported to be destructive on cider apples in Normandy. Today, the disease is not considered of economic importance in most years, and has not been reported from the dry region of south-eastern France (J.-P. Paulin, personal communication).

The first fire blight infections in Belgium were reported by Veldeman (1972) near the coast of the North Sea. Soon the disease became quite severe on the pear cultivar 'Conference' and losses of 300–400 trees were documented. Deckers (1996) reported that it took fire blight 5 years to spread from the coastal province of West Flanders to the eastern province of Limburg, a distance of 200 km. In Belgium, the most susceptible pear cultivar proved to be 'Durondeau', followed by 'Doyenne du Comice' and 'Conference' (Deckers, 1996). As has been observed in other countries, the first apple cultivars did not become infected until 10 years after the first appearance of fire blight on pears (van der Zwet and Keil, 1979). The common hawthorn hedges and extremely susceptible *Cotoneaster salicifolius floccosus* were very instrumental in the spread of the bacterium to fruit orchards (Deckers, 1996). The economic impact of one blight incidence on 'Braeburn' apple trees in a nursery resulted in the removal of 20,000 trees at a cost of \$70,000, not including the labour (T. Deckers, personal communication). Fire blight moved steadily eastward and was reported in Luxembourg by 1982 (EPPO, 1983).

Fire blight was first reported in Poland from the coastal region of the Baltic Sea near Milobadz, about 25 km south of the port city of Gdansk (Borecki *et al.*, 1967). The disease, observed in the same year (1966) as the first recording in The Netherlands, was most probably overlooked along the coastal and island regions between these two countries. By the end of the growing season, the disease was well established in several apple and pear orchards and *E. amylovora* was positively identified (Borecki and Lyskanowska, 1968). By 1985, fire blight had spread further south and inland towards the centre of the country and had affected hawthorn hedges and numerous susceptible apple cultivars (Sobiczewski and Suski, 1988).

Fire blight appeared on the southern islands of Denmark in 1968 on hawthorn and slowly spread to the neighbouring islands in the next 5 years. Klarup (1969) published a detailed account of the initial outbreaks, including

eradication measures. After several years, the amount of fire blight on pears was significantly reduced when hawthorns were kept at least 25 m away from fruit trees (Bech-Andersen, 1973). During the period 1980–1995, fire blight moved slowly but steadily eastward and northward from the infested areas in north-western Europe. By 1986, the disease was reported on pear from southern Sweden (Graeborg, 1993) and on cotoneaster from the west coast of Norway near Stavanger (Sletten, 1990). In the three Scandinavian countries, fire blight never reached losses of economic importance.

Since its appearance in Germany in 1971, fire blight has moved steadily across the country and eventually has become more severe in the warmer, more humid portions of the south (near Lake Constanzt) than in the much cooler north. A comprehensive review of the early history was published by Zeller (1974). The disease has been reported to be severe on apple cultivars 'Gloster', 'Jonathan' and 'James Grieve', as well as on hawthorn hedges and the most attractive but highly susceptible *C. salicifolius*. Fire blight is regarded as an economic problem in the fruit-growing areas of Baden-Wurttemberg and Rheinland-Pfalz in the south (W. Zeller, personal communication). In the latter region, 200 ha of fruit trees were eradicated in each of the years 1993, 1995 and 1996. In the meantime, fire blight spread across all of Germany and reached the Czech Republic by 1987 (Kudela, 1988) and Switzerland by 1989 (Grimm, 1989). The first report from western Austria appeared in 1993 (Keck *et al.*, 1996). In the latter two countries, the disease has remained fairly localized.

During the time that fire blight emerged in the UK and appeared on the European continent, the disease also appeared in the north-east corner of the African continent. In 1964, El-Helaly *et al.* (1964) reported fire blight near the port city of Alexandria in the Nile delta of Egypt. The disease had been observed for at least 2 years in several governorates and all recordings were made on the low-chilling pear cultivar 'Le Conte'. The disease remained rather mild in the early years, and for about 6 years (1966–1972) it appeared that fire blight had been eradicated (El-Goorani, 1973). But, during the early 1980s, the disease became very severe, presumably due to an increase in rain during the blooming period (K.Y. Mickail, personal communication). Numerous cankers showed the papery bark symptom, characteristic of fire blight, in *Pyrus* germplasm with *P. pyrifolia* parentage, and the disease was officially identified as fire blight, through the application of fatty acid profiles (van der Zwet, 1986). By 1988, 80% of all pear acreage was affected and 50% of all trees had been eradicated; however, the disease was never observed in the large desert of Faiyum. Considering the land rent and all agricultural practices involved, the loss of 0.4 ha (160 trees) or a feddan due to fire blight is estimated at \$4000 (M.K. El-Kazzaz, personal communication).

On the island of Cyprus, the disease showed up on many trees and in numerous locations. The first report in 1986 indicated that fire blight may have been present for several years (Psallidas and Dimova, 1986). A survey in 1985 reported nearly 28,000 infected pear trees and 16,000 apple trees infected with fire blight, of which 29,000 trees were destroyed, the equivalent of 100 ha of

trees. The disease was especially severe on the pear cultivar 'Beurre Superfine', which constituted about 90% of the pear acreage in Cyprus. By 1986, fire blight was reported from all pear- and apple-producing areas on the island and some cultivars ('Beurre Superfine' pear and 'Pera Pedit' apple) were totally destroyed. In 1988, 8300 severely blighted apple and pear trees were uprooted at a farmer subsidy cost of \$133,000 (P.G. Psallidas, personal communication).

In May 1985, severe fire blight was observed in Israel in seven different orchards, four in the north and three in the southern part of the country (Zutra and Shabi, 1985). Hundreds of trees were affected, mainly in the cultivars 'Spadona', 'Gentile' and 'Coscia', the three principal cultivars grown in Israel (Shabi and Zutra, 1987). In 1986, the disease was detected in eight new sites scattered all over the country, including one pear orchard in the Negev Desert in the south (Shabi and Zutra, 1987). In Israel, quince (*Cydonia*) and loquat (*Eriobotrya japonica*) are important and sensitive hosts to fire blight (Zilberstaine *et al.*, 1996).

Once fire blight became established in the Egypt–Cyprus–Israel triangle, it was only a matter of time before the disease appeared in neighbouring countries. In 1985, fire blight was reported from the coastal areas of Turkey and the following year from the island of Crete in Greece (EPPO, 1987). In Turkey, the disease spread rapidly across the country from west to east and was particularly severe on quince and medlar (*Mespilus* spp.) (Benlioglu, 1988). By 1988, the disease was reported on pear in Lebanon (EPPO, 1988) and, by 1990, on quince in Jordan (Tehabsim *et al.*, 1992). That year (1990), the disease had spread across to eastern Turkey and was reported from Armenia (Ministry of Agriculture, personal communication). From eastern Turkey, the disease moved into Iran and, by 1995, fire blight was reported in the central and north-western parts of the country (Afunian and Rahimian, 1996). They reported that the biological characteristics of 50 isolates of *E. amylovora* were quite homogeneous.

From Crete and Turkey, fire blight moved northward into the Peloponnese and mainland areas of Greece and from there into Macedonia, Bulgaria and Romania. In Greece, the disease quickly became a serious problem all over the country, especially on the local pear cultivar 'Kontoula' (Psallidas, 1990). Psallidas also reported that, in one location, 30 of 45 ha were totally uprooted and, by 1988, 300 ha were destroyed (P.G. Psallidas, personal communication). In Macedonia, the disease was very destructive to pear and quince (Mitrev, 1996). Costs of eradication procedures, including destruction of affected trees and planting new trees, were reported as \$7 million. In Bulgaria, fire blight was first found near Plovdiv and several years later in the region of Kjustendil near the border of Macedonia (Bobev, 1990). The first survey in Romania indicated that the disease had been present at least 1 year earlier (Baicu *et al.*, 1994). Several local apple and pear cultivars were severely affected. Soon after Macedonia, fire blight was also reported in Yugoslavia (Serbia, Bosnia, Croatia) (Panic and Arsenijevic, 1993) and Albania (Pace and Mazzucchi, 1996). By 1996, fire blight appeared in south-eastern Hungary, where the disease probably became established in 1993 or 1994 but was first observed in early 1996 (Hevesi, 1996). By the end of that year, the following numbers of trees and

shrubs were eradicated by the Ministry of Agriculture at a total cost of \$1.1 million: 47,000 apples, 8600 quince, 8100 pear, 1000 medlar and 600 various ornamentals (J. Nemeth, personal communication).

Once fire blight was established throughout the southern Balkans, it came as no surprise that symptoms had been observed on pear trees in the southernmost part (heel of the boot) of Italy in the Apulian provinces near Lecce (Cariddi and Piglionica, 1992). The following year, the disease was observed on the island of Sicily (d'Anna *et al.*, 1994). In spite of intensive efforts through the maintenance of a sophisticated prediction and monitoring system in the Po River valley in northern Italy, fire blight was detected near the city of Bologna in 1994 (Calzolari *et al.*, 1999). From all indications, it appeared to have been introduced through contaminated bud wood to a fruit-tree nursery. In summer 1997, more than 2400 ha of pear trees became severely infected following a hailstorm (C. Bazzi, personal communication).

In 1996, the first report of fire blight appeared in northern Spain (de la Cruz Blanco, 1996). The disease was observed in August 1995, mainly on cider apples, a few kilometres south of the French border.

Conclusion/summary

In summary, since the late 1700s, the fire blight disease has managed to spread to 39 additional countries from its original location in eastern New York (Table 3.1). It may be present in additional countries, but not yet observed or still unreported. After the disease spread across North America, the dissemination to the remaining 36 countries was instigated solely by humans through the long-distance shipment of contaminated bud wood or trees. Apart from the long-distance movement to Japan, New Zealand and Bermuda, the occurrences in the remaining 33 countries appear to have resulted from its introduction into two separate countries – England and Egypt – both within a few years following the Second World War. After 45 years of incremental short-distance spread, these introductions have culminated in the presence of fire blight in nearly every country in Europe and the Middle East. Following its spread into regions of varying climatological conditions, it has become obvious that disease is considerably more severe in warm, humid areas than in cooler and/or dry areas. Countries in northern versus southern Europe follow the same pattern of disease severity and economic impact as do the northern versus southern states of the USA.

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Genetic Diversity and Host Range of *Erwinia amylovora*

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Introduction

Management of fire blight caused by *Erwinia amylovora* (Burrill) Winslow *et al.* is a continual challenge to growers and extension specialists, and particularly to researchers. One of the reasons for limited success in disease management is an inadequate understanding of the host range and genetic diversity of strains of *E. amylovora*. The extent and the molecular nature of the diversity of the pathogen and its relationship to epidemiology and to host–pathogen interaction are not fully understood. However, recent results using molecular techniques have greatly advanced our knowledge of genetic diversity within *E. amylovora* strains, providing levels of precision not previously available (McManus and Jones, 1995; Momol *et al.*, 1995, 1997; Zhang and Geider, 1997).

Characterization of strain diversity and host range in *E. amylovora* is primarily important in plant quarantine, selection for disease resistance in breeding and genetic engineering programmes, tracking long- or short-range pathogen dispersal, identifying possible sources of infection, distinguishing strain groups and studying the relatedness among strains.

This review seeks to summarize some of the findings of host range and strain diversity studies on *E. amylovora* that have been made, mainly during the last 10 years. It is not meant to be an exhaustive review of the literature but rather a reflection on some recent techniques that have produced new findings about the genetic diversity of *E. amylovora*.

Host range

In general, strains of *E. amylovora* are not host species-specific. For example, most strains of *E. amylovora* isolated from apple (*Malus domestica*) are also pathogenic on pear, on other *Maloideae* and on *Prunus* (van der Zwet and Keil, 1979; Mohan and Thomson, 1996). Interestingly, strains of *E. amylovora* isolated from *Rubus* species are not pathogenic on pear (*Pyrus communis*) or apple (Starr *et al.*, 1951; Ries and Otterbacher, 1977; Heimann and Worf, 1985). Strains isolated from Asian pear in Hokkaido, Japan, also show some host specificity. They are as pathogenic to European pears as North American strains isolated from *Maloideae*, but exhibit an incomplete pathogenicity toward apple cultivars (Beer *et al.*, 1996). Another notable report concluded that some cultivars of apple were highly resistant to strain Ea273 but fully susceptible to strain E4001A (Norelli *et al.*, 1986).

E. amylovora is known as a pathogen of *Rosaceae* and has a wide host range within that family. Van der Zwet and Keil (1979) summarized a diversity of reports about fire blight inducing symptoms on ~200 species in 40 rosaceous genera. Some of these reports were based on artificial inoculations, which resulted in symptoms that may have been due to incompatible reactions rather than compatible reactions.

Natural infection by *E. amylovora* is not limited to species belonging to the *Maloideae* subfamily of *Rosaceae*. Natural infections of *Rubus* spp. (*Rosoideae*) (Starr *et al.*, 1951; Evans, 1996) and *Prunus salicina* (*Amygdaloideae*) (Mohan and Thomson, 1996) are good examples of non-*Maloideae* host plants.

The *Rosaceae* are divided into four subfamilies, each with a distinct fruit type: fruits from the *Spiraeoideae* subfamily have follicles, *Rosoideae* produce achenes or drupels, *Amygdaloideae* produce drupes and *Maloideae* produce pomes. Twenty-eight genera have been recognized in the *Maloideae* subfamily (Robertson *et al.*, 1991).

Maloideae (*Pomoideae*)

Fire blight owes most of its economic importance worldwide to its effects on members of this subfamily, especially pear and apple. Fire blight can, however, damage other *Maloideae* fruit crops. Recently, serious outbreaks of fire blight on quince (*Cydonia*) and loquat (*Eriobotrya japonica*) were reported from Turkey (Momol and Yegen, 1993) and Israel (Zilberstaine *et al.*, 1996).

Many ornamental plants in the *Maloideae* are hosts of *E. amylovora*. Infection on blossoms and twigs after artificial inoculation was observed on Japanese quince (*Chaenomeles lagenaria*) by Rosen and Groves (1928). Van der Zwet and Keil (1979) cited many reports of fire blight on mountain ash (*Sorbus*), hawthorn (*Crataegus*), firethorn (*Pyracantha*), *Cotoneaster*, *Stranvaesia*, *Photinia* and service berries (*Amelanchier*).

Rosoideae

E. amylovora infects raspberry and blackberry (bramble) (*Rubus*) plants, which belong to the *Rosoideae* subfamily. Strains isolated from *Rubus* spp. were found to be host-specific and did not infect apple or pear (Starr *et al.*, 1951; Ries and Otterbackher, 1977; Heimann and Worf, 1985). Blossoms and young twigs of 'Fairfax' rose (*Rosa* sp.) are found to be very susceptible to *E. amylovora* in artificial inoculation tests (Rosen and Groves, 1928). Other hosts cited by van der Zwet and Keil (1979) that belong to the *Rosoideae* are the ornamentals cliff rose (*Cowania*), cinquefoil (*Potentilla*), avens (*Geum*) and *Dryas*.

Amygdaloideae (*Prunoideae*)

E. amylovora was found to infect *P. salicina* (Japanese plums) a long time ago (Rosen and Groves, 1928), but recently an extensive outbreak of fire blight on *P. salicina* in Idaho was reported by Mohan and Thomson (1996). Strains isolated from this recent outbreak and a known isolate of *E. amylovora* from apple caused the same symptoms on apple and Japanese plums as observed in original infections. Based on cultural and physiological tests, inoculation of plum, apple and pear, cellular fatty acid profiles, and pEA29 fragment PCR, *Prunus* strains were not different from an apple strain (FB93-1) of *E. amylovora*.

Spiraeoideae

Artificial inoculation of young twigs of *Spiraea vanhouttei* resulted in a compatible reaction, with symptoms similar to frost injury (Rosen and Groves, 1928). Ninebark (*Physocarpus*), goatsbeard (*Aruncus*) and cream-bush (*Holodiscus*) were also cited as hosts of *E. amylovora* (van der Zwet and Keil, 1979).

Reservoir hosts

E. amylovora has a wide host range and in some regions may overwinter on plants which might not be the most economically important host and are called reservoir hosts. *E. amylovora* commonly overwinters on *Pyracantha* spp. in California, and populations of the bacterium in the spring are often as great as 10^5 cells per flower (Thomson *et al.*, 1975). These flowers constitute a source of inoculum, which can lead to the infection of secondary pear flowers (Schroth *et al.*, 1974). Very susceptible quince trees appear to be a significant source of inoculum for neighbouring pear orchards in the Korkuteli area, Antalya, Turkey. In England, hawthorn appears to be the major overwintering host (Glasscock, 1971; Billing, 1978), and diseased hawthorn hedges have sometimes been important sources of fire blight inoculum for pear and apple trees in English orchards (Berrie and Billing, 1996). Any reservoir host in proximity to apple or pear orchards and nurseries should be eliminated where possible. The

reservoir host is particularly dangerous if its bloom precedes the crop host's and allows build up of inoculum that can subsequently infect the crop host's blossoms.

Specialization of *E. amylovora* strains toward the host

Biovars based on biochemical and physiological criteria have thus far not been described formally in *E. amylovora*, nor have pathovars based on pathogenicity to a particular host species. However, there are two reports in the literature of the taxonomic designation of *E. amylovora* strains based on pathogenicity to particular hosts.

The first is by Starr *et al.* (1951), who suggested naming certain strains *E. amylovora* f. sp. *rubi*, based on pathogenicity to *Rubus*. The brambles, botanically known as the genus *Rubus*, contain an amazing range of plant forms. More than 740 species, many of which reproduce non-sexually, have been described throughout the world and variously classified into 12 or 15 subgenera (Hummer, 1996). This diverse group of hosts is affected by only a limited number of strains in the largest collections of *E. amylovora* strains, and yet two distinct groups of *Rubus* strains have been reported by several studies accomplished by different methods (Laby and Beer, 1992; Kim *et al.*, 1995, 1996; McManus and Jones, 1995; Momol *et al.*, 1995, 1997). Recently, *E. amylovora* isolated from 'Westland' apple caused black lesions and ooze on 'Boyne' raspberry shoots following artificial inoculation (Evans, 1996). However, one *E. amylovora* strain isolated from naturally infected 'Boyne' raspberry did not cause infection on apple (Evans, 1996).

The second report of strains of *E. amylovora* affecting specific hosts is in a book written by Goto in 1990 (English translation 1992). On Hokkaido, Japan, the disease named 'bacterial shoot blight of pear' (BSBP) was reported on twigs of the Asian pear (*Pyrus pyrifolia* (Burm. f.) Nak.) cultivar 'Mishirazu', causing blight of blossoms, young fruits, leaves and shoots. Bacterial strains isolated from Mishirazu infected certain Asian pears, but were reported not to infect cultivars of apple. This suggested that there are some significant biological differences between those strains and other strains of *E. amylovora*. Furthermore, Goto stated that 'it is considered to be a distinct pathovar of *E. amylovora*'. Recent results indicate that BSBP should properly be called fire blight and is caused by strains of *E. amylovora* that differ by several criteria, including host ranges, from strains isolated from *Maloideae* in other countries (Beer *et al.*, 1996). Except for the oldest isolated strain, strains isolated from Hokkaido caused the usual incidence and severity of infection on shoots of European and Asian pear, similar to those of *E. amylovora* strains from *Maloideae* in North America. In contrast, many apple cultivars are not infected by *E. amylovora* strains isolated in Hokkaido, several cultivars are infected at low frequency and a few (mainly 'Jonathan' sports) are infected at high frequency. One of the differentiating host range characteristics of Hokkaido strains is the limited range

of pathogenicity to apple cultivars. Random amplified polymorphic DNA (RAPD) analysis of *E. amylovora* strains distinguished all three major groups of strains (Momol *et al.*, 1997) described above which show some host specialization: *Maloideae* strains not pathogenic to *Rubus*, *Rubus* strains only pathogenic to *Rubus*, Hokkaido strains pathogenic to pear and of limited pathogenicity toward apple.

Virulence and differential virulence

J.C. Arthur (1887) was the first to report the diversity of strains of *E. amylovora*. He observed differences in blight infections when 'Bartlett' and 'Seckel' cultivars of pears were inoculated with *E. amylovora*. Marked variability in virulence of some *E. amylovora* strains was noted and correlated with morphological and some physiological characteristics (Ark, 1937). Other studies also showed that strains may vary in virulence (Pierstorff, 1931; Shaffer and Goodman, 1962).

Differential virulence in strains of *E. amylovora* has been described on apples. *E. amylovora* strain Ea273 was found to be pathogenic on most apple cultivars but caused little or no disease on apple cultivars 'Quinte', 'Ottawa 523' and 'Novole', and on *Malus* × *robusta* No. 5, whereas strain E4001A was fully virulent to these cultivars (Norelli *et al.*, 1984, 1986).

Strain diversity

A strain is made up of the descendants of a single isolation in pure culture and is usually derived from a single colony (Lapage *et al.*, 1975). Several studies have indicated that strains of *E. amylovora* form a homogeneous group (Billing *et al.*, 1961; Komagata *et al.*, 1968; Paulin and Samson, 1973; Paulin, Chapter 6). In the past, no characteristics have been found that could distinguish strains of different geographical origins or strains that have been isolated either from different host plants or at different times (Vanneste, 1995). Based on detailed taxonomic studies of the genus *Erwinia*, but using only *Maloideae* strains, Dye (1968) found no major differences in biochemical characters or carbohydrate utilization among members of the *E. amylovora* group. Again, in the absence of *Rubus* and Hokkaido strains, based on a serological study, Elrod (1941) concluded that *E. amylovora* was an exceedingly homogeneous species. Vantomme *et al.* (1982) tested 103 isolates of *E. amylovora* and found them to be quite homogeneous in their biochemical and protein electrophoretic characteristics, despite their different geographical and host origins.

E. amylovora is not a diverse species like *Pseudomonas syringae* (Hirano and Upper, 1990) or *Xanthomonas campestris*, but with the help of molecular techniques the genetic diversity in *E. amylovora* strains is now being better characterized. Such diversity at a molecular level could be used to monitor development of pathogenic strains in space and time, to identify possible sources

of infection, to assist in gene mapping, to aid in individual strain identification, to study the population genetics of species and to serve as characters in molecular phylogenetic studies (Bowditch *et al.*, 1993).

Early evidence of strain diversity and its importance in the understanding of epidemiology of fire blight were summarized, based on the review by Schroth *et al.* (1974). Strains vary in virulence, colony morphology (Fig. 4.1), serology, phage typing and sensitivity to antibiotics (Paulin, Chapter 6). Streptomycin-resistant strains of *E. amylovora* could be placed in three major phenotypes, based on their level of sensitivity to streptomycin (McManus and Jones, 1995; Jones and Schnabel, Chapter 12).

Biochemical tests, serology and fatty acid profile

Several biochemical tests can be performed to differentiate strains of *E. amylovora*. Schwartz *et al.* (1991) reported that synthesis of dihydrophenylalanine (DHP) was not equivalent for 12 *E. amylovora* strains they studied and their effect on agar-embedded pear cells was different. Only three strains out of 12 (from the USA) were found to produce DHP.

Distinct groups of *E. amylovora* were identified based on carbon utilization, as determined with the BIOLOGTM system (Kim *et al.*, 1996). The carbon source utilization profiles of each strain were compared by a cluster analysis. Most

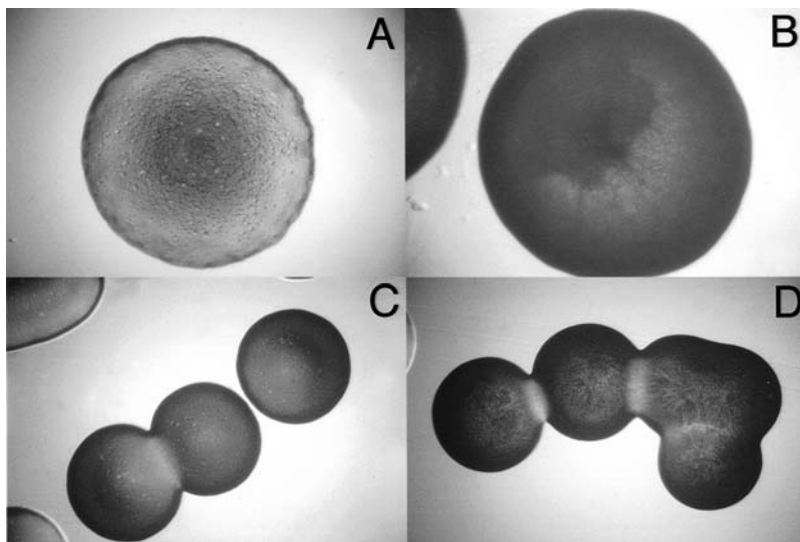


Fig. 4.1. Colony morphology of strains *Erwinia amylovora* Ea273 from apple (plate A), E4001A from apple (plate B), Ea528 from *Rubus* (plate C) and Ea562 from Asian pear on Hokkaido, Japan (plate D) (magnification $\times 10.5$). Bacteria were incubated for 48 h at 28°C on modified MS (MMS) medium (Brulez and Zeller, 1981).

strains isolated from *Maloideae* hosts, excluding those from Hokkaido, clustered together. At least two different groups were recognized for strains pathogenic on *Rubus* spp. Strains isolated from Hokkaido formed two distinct groups. Serological tests can distinguish Hokkaido strains from *Maloideae* strains (Steven Beer, Ithaca, New York, USA, personal communication). Except for strains isolated from *Rubus* plants, strains isolated from different geographical origins and from different hosts were similar to each other in respect of percentage of fatty acid classes (van der Zwet and Wells, 1993). *Rubus* strains showed a slight increase in cyclic acids compared with the other strains.

pEA29-PCR

With very few exceptions, all strains of *E. amylovora* carry a similar plasmid of c. 29 kb called pEA29 (Vanneste, 1995). This characteristic allows the identification of *E. amylovora* by PCR amplification of a 0.9 kb fragment from this plasmid (Bereswill *et al.*, 1992). The sensitivity of detection of *E. amylovora* cells using PCR has been improved with amplification by nested PCR of the 0.9 kb *Pst*I fragment of pEA29, which, after sequencing, has been found to be slightly larger in size (1 kb) than previously reported (McManus and Jones, 1995).

The PCR product using the primers derived from pEA29 has been used to distinguish several strains of *E. amylovora*. All of the *Maloideae* strains, except those from Hokkaido, and most of the *Rubus* strains produced a 1 kb (expected size) PCR product. One *Rubus* strain and all strains from Asian pear from Hokkaido produced slightly larger bands of sizes 1.1–1.2 kb (Kim *et al.*, 1995). We used the primers A and B derived from pEA29 (Bereswill *et al.*, 1992) for detection of *E. amylovora*, using a modified PCR procedure (Momol *et al.*, 1994). In a RAPD fingerprinting study (Momol *et al.*, 1997), all strains of *E. amylovora* isolated from *Maloideae* (except those from Hokkaido), *Rubus* and Asian pear on Hokkaido were confirmed to be *E. amylovora* by PCR. However, product size larger than 1 kb was detected for some *Rubus* and all known Hokkaido strains (Fig. 4.2). This simple PCR procedure may be used to distinguish some *Rubus* and all Hokkaido strains from non-Hokkaido *Maloideae* strains as a simple fingerprinting of *E. amylovora* strains. In conjunction with a pathogenicity test on *Rubus*, three major groups of *E. amylovora* may be distinguished from each other by pEA29-PCR amplification.

In a recent study, DNA from 127 strains of *E. amylovora* was amplified by PCR, using as primer a fragment of the plasmid pEA29 (Lecomte *et al.*, 1997). As observed previously (Kim *et al.*, 1995, 1996; Beer *et al.*, 1996; Momol *et al.*, 1997), a larger than expected size of DNA fragment was obtained for some strains. After digestion of the PCR products by *Msp*I and *Sau*3A, three distinct groups of banding patterns were observed. Group 3 appeared to be restricted to the strains from a recent fire blight outbreak of *E. amylovora* in the specific geographical area of Lake Constance, southern Bavaria (Germany and western Austria).

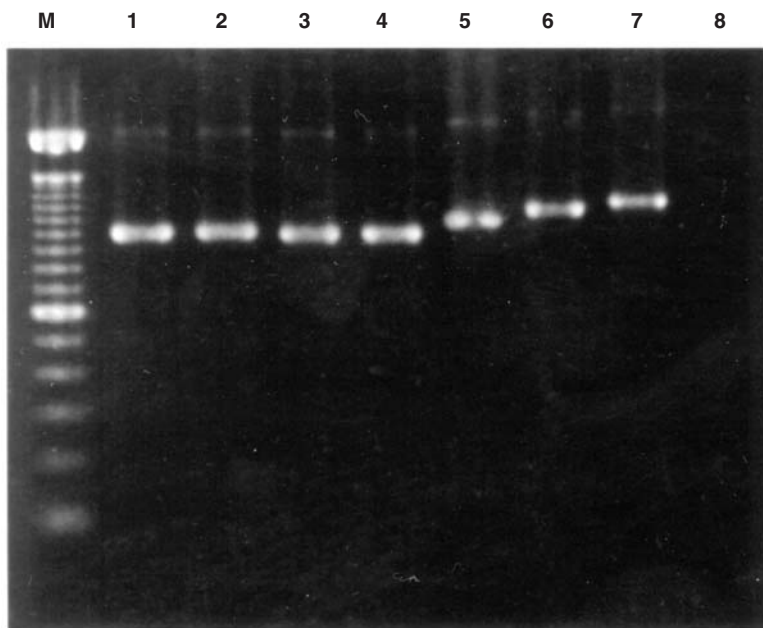


Fig. 4.2. pEA29-PCR (Bereswill *et al.*, 1992) amplification products (1–1.2 kb) generated for the strains listed below demonstrate differentiation among some strains. Lane designations: **M**, 100 bp DNA marker; **1**, E4001A (apple); **2**, Ea273 (apple); **3**, Ea416 (*Rubus*); **4**, Ea528 (*Rubus*); **5**, Ea510 (*Rubus*); **6**, Ea546 (Asian pear, Hokkaido, Japan); **7**, Ea 556 (Asian pear, Hokkaido, Japan); **8**, water.

Repetitive element PCR and PCR ribotyping

Bacteria commonly carry several copies of a number of repetitive DNA sequences, such as repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX sequences. Repetitive element PCR (rep-PCR) takes advantage of the presence of these DNA sequences to identify bacterial strains or species. Primers are designed from each half of the conserved repeat element and directed outward. Agarose gel electrophoresis of the PCR products obtained results in a fingerprinting pattern according to the sizes of DNA fragments amplified between individual repetitive elements on the bacterial genome (Versalovic *et al.*, 1991). Size polymorphism of the 16S–23S spacer region among different strains (PCR ribotyping) has also been used for *E. amylovora* by McManus and Jones (1995) and Jeng *et al.* (1999).

Rep-PCR and PCR ribotyping were determined for 189 strains of *E. amylovora* strains isolated from *Maloideae* and *Rubus* hosts from Canada, the USA and New Zealand (McManus and Jones, 1995). A total of 115 strains (61% of the strains in this study) were isolated from apple or pear in Michigan.

Even though the strains came from a geographically compact area and from a narrow host range, significant differences in rep-PCR fingerprinting band patterns were observed using the REP, ERIC and BOX primers. The most striking differences occurred between tree fruit (*Maloideae*) and *Rubus* strains (McManus and Jones, 1995). In the same fingerprinting study, *E. amylovora* strains were placed into four distinct PCR ribotyping groups.

***hrp* gene restriction fragment length polymorphism (RFLP)**

Most plant-pathogenic bacteria possess a cluster of genes called *hrp* genes, which is necessary for induction of hypersensitivity on non-host plants and for pathogenicity on host plants (Kim and Beer, Chapter 8). The *hrp* gene cluster of *E. amylovora* strain Ea321 was used as a probe to detect potential sequence variation in the *hrp* cluster of *E. amylovora* strains isolated from *Maloideae* and *Rubus* plants and from diverse geographical origins (Laby and Beer, 1992). Under high-stringency conditions, the composite Ea-*hrp* probe hybridized only with DNA derived from *E. amylovora* and not with DNA from other organisms. All *Maloideae* *E. amylovora* strains in this study yielded hybridization patterns identical to that of Ea321. DNA from strains isolated from *Rubus* spp. yielded two hybridization patterns distinct from the patterns obtained from *E. amylovora* strains that had been isolated from *Maloideae* hosts (Laby and Beer, 1992). In another study using *hrp* gene RFLP, five different patterns were discernible: one for *Maloideae* strains, two for *Rubus* strains and two for strains from Asian pear on Hokkaido (Kim *et al.*, 1996).

Random amplified polymorphic DNA (RAPD) fragment analysis

DNA fingerprinting involves the display of a set of DNA fragments from a specific DNA sample. A variety of DNA fingerprinting techniques are currently available. RAPD (Williams *et al.*, 1990) and arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990) fragment analyses have been used as sensitive and efficient methods for distinguishing different strains of several bacteria, including *Escherichia coli* and *Helicobacter pylori* (Berg *et al.*, 1994).

For plant-pathogenic prokaryotes, RAPD analysis has been used to study genetic relatedness among mycoplasma-like organisms associated with several geographically diverse grapevine yellows diseases (Chen *et al.*, 1994), but also to determine phylogenetic relationships within *X. campestris* (Smith *et al.*, 1994), as well as to distinguish strains of *X. campestris* pv. *pelargonii* from 21 other *Xanthomonas* species and/or pathovars (Manulis *et al.*, 1994), and to detect differences between isolates of *P. syringae* pv. *apii* isolated from California or eastern North America (Little *et al.*, 1994). RAPD analysis was also used to reveal genetic and phenotypic variation of *Agrobacterium* biovars (Irelan, 1994).

Verification of strain identity of *Agrobacterium vitis* was achieved by RAPD analysis of total genomic DNA (Burr *et al.*, 1995). RAPD analysis has also provided markers to differentiate races of several plant pathogenic fungi (Assigbetse *et al.*, 1994).

Momol *et al.* (1995, 1996, 1997) used RAPD analysis to unambiguously identify ('fingerprint') and determine the relatedness of 16 different strains of *E. amylovora*, as well as to determine whether RAPD data support the grouping of strains based upon the host plants or geographical origins from which they were isolated. Twenty-four 10-mer arbitrary primers were screened, and RAPD fragments produced by six of the primers were chosen for RAPD analysis. Genetic similarities between strains and groups of strains were computed by the method of Nei and Li (1979) from presence/absence of RAPD fragments. To determine relationships between strains or groups of strains, these were used in the unweighted pair group method using arithmetic means (UPGMA) cluster analysis procedure (Sneath and Sokal, 1973), an option of the computer program NTSYS-pc (Rohlf, 1988). Using these techniques, Momol *et al.* (1995, 1997) found genetic diversity within a collection of 16 *E. amylovora* strains isolated from the USA, Europe and Japan. Strains were classified into three RAPD groups: *Maloideae*, *Rubus* and 'Hokkaido' (Asian pear from Hokkaido, Japan) (Fig. 4.3). *Rubus* and Hokkaido groups formed two subgroups. Therefore, all strains in the RAPD study could be classified into five distinct groups and subgroups: *Maloideae* (apple and pear strains from the USA and Europe), *Rubus* I (Ea416), *Rubus* II (Ea528 and Ea510), Hokkaido I (7971(1)) and Hokkaido II (TP9405). The grouping of strains by RAPD is consistent with the finding of Kim *et al.* (1996, 1999), who grouped *E. amylovora* strains based on their utilization of carbon sources, as determined by the BIOLOGTM system, *hrp* gene RFLP, pEA29 PCR and cleaved amplified polymorphic sequences (CAPS)

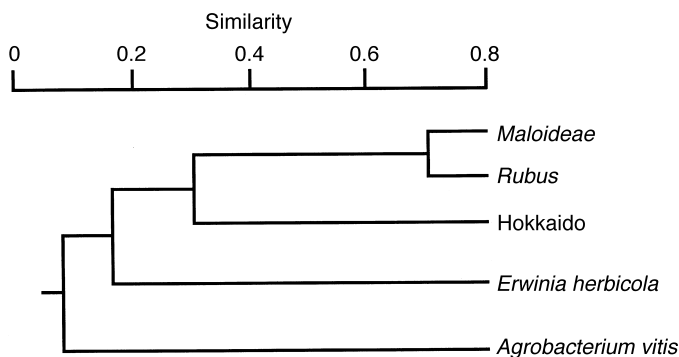


Fig. 4.3. Phenogram from UPGMA clustering of three major groups (*Maloideae*, *Rubus*, Hokkaido) of *Erwinia amylovora* strains based on RAPD fingerprinting analysis (Momol *et al.*, 1997). *Erwinia herbicola* and *Agrobacterium vitis* were used as outgroups.

analysis. RAPD analysis has been used successfully to facilitate the identification of reisolated Hokkaido strains from apple (Beer *et al.*, 1996).

AP-PCR analysis (Welsh and McClelland, 1990), a very similar technique to RAPD analysis (Williams *et al.*, 1990), was used to identify *E. amylovora* by Bereswill *et al.* (1995) but failed to distinguish between five *Maloideae* strains isolated from Egypt, Germany, Turkey and the USA.

Pulsed-field gel electrophoresis (PFGE)

Genomic DNA digested with rare-cutting endonuclease enzymes can be separated by PFGE, generating PFGE patterns. PFGE patterns of *E. amylovora* strains isolated from different geographical regions showed that strains from some geographical areas were related. The banding patterns obtained by *Xba*I digestion revealed significant differences among strains from different areas. North American and English strains had the most divergent PFGE patterns. Strains from Egypt, Greece and Turkey were found to be closely related (Zhang and Geider, 1997). This technique may be useful to trace the origin of the pathogen after new outbreaks of fire blight (Zhang *et al.*, 1996). The spread of fire blight in the eastern Mediterranean and the Balkans since 1982 was mapped based on the reported PFGE pattern of *E. amylovora* strains in different countries (Bazzi *et al.*, 1999). Previous epidemiological analysis suggested that the dissemination of *E. amylovora* in the eastern Mediterranean area originated in the Nile delta of Egypt (Momol and Zeller, 1992). PFGE findings regarding strains isolated from Egypt, Greece and Turkey (Zhang and Geider, 1997; Bazzi *et al.*, 1999) support this hypothesis. Based on PFGE analysis, strains from northern Italy overlap with French strains and strains from southern Italy with the Mediterranean pattern (Merighi, 1996; Bazzi *et al.*, 1999).

Amplified ribosomal DNA restriction enzyme analysis (ARDREA)

ARDREA (Selenska-Pobell *et al.*, 1998), also called ribofingerprinting (Burr *et al.*, 1995), was used previously to characterize *A. vitis* strains (Otten *et al.*, 1996; Momol *et al.*, 1998). For ARDREA, the 16S–23S intergenic spacer (IGS) region of *E. amylovora* DNA was amplified and digested with several restriction enzymes. ARDREA has been used to fingerprint the *E. amylovora* strains isolated in Australia (Kim *et al.*, 1999).

Grouping of *E. amylovora* strains based on ARDREA (Momol *et al.*, 1999) and RAPD (Momol *et al.*, 1997) were similar, except for the Hokkaido strains, which did not cluster into a group by ARDREA.

Cleaved amplified polymorphic sequences (CAPS)

A method similar to ARDREA was developed (E.R. Garr, D.W. Bauer and S.V. Beer, manuscript in preparation) to identify the different groups of *E. amylovora*. This procedure, called CAPS, involves the PCR amplification of the *hrp* gene cluster, followed by digestion with restriction endonucleases. Restriction enzymes that generate distinct fragments for each group of *E. amylovora* were identified (Kim *et al.*, 1999). In their CAPS analysis study, Kim *et al.* (1999) designated the different groups of *E. amylovora* strains as 'M' for the type strains for *Maloideae* strains, 'RI' and 'RII' for the type strains for *Rubus* and 'P' for the type strains for 'Hokkaido' strains.

Conclusion/summary

As fire blight spreads through the world's apple- and pear-growing regions and intensifies in endemic areas as more susceptible cultivars and rootstocks are grown, the need for a better understanding of the genetic diversity and host range of *E. amylovora* strains becomes even greater. Implementation of intelligent quarantine protocols is fully dependent on a comprehensive knowledge of strain diversity and the identification of strains to group. Selection of resistant cultivars and rootstock in breeding and genetic engineering programmes must take account of the range of diversity for pathogenicity in *E. amylovora*, so that resistance will be durable and universally effective. Strain identification is also necessary for understanding the dispersal and spread of the pathogen.

The majority of known strains of *E. amylovora* were isolated from *Maloideae* plants and in this review were referred to as *Maloideae* strains. Strains from Hokkaido, Japan, were isolated from Asian pear and formed a distinct group of strains, based on RAPD analysis (Momol *et al.*, 1995, 1997), serology, pathogenicity, PCR amplification of a fragment from pEA29 and some biochemical and molecular characteristics (Kim *et al.*, 1995, 1996; Beer *et al.*, 1996). The third group was strains isolated from the *Rosoideae* subfamily, mainly from *Rubus* spp. Other strains were isolated from *Prunus* in the *Amygdaloideae* subfamily, but these strains did not show any features that distinguished them from the apple strain. Pending further characterization with DNA fingerprinting techniques, they should remain in the *Maloideae* group. At the host subfamily level, host range plays an important role, especially in the main differences between *Maloideae* and *Rosoideae* strains. But, even within *Malus*, different cultivars reacted differently with different strains of pathogen (differential virulence) (Norelli *et al.*, 1984, 1986). Some differences among *Maloideae* strains were detected by RAPD analysis (Momol *et al.*, 1995, 1997), ribotyping (McManus and Jones, 1995), pEA29 fragment restriction enzyme fingerprinting (Lecomte *et al.*, 1997) and PFGE (Zhang and Geider, 1997). PFGE (Merighi, 1996; Zhang and Geider, 1997) and pEA29 fragment restriction enzyme fingerprinting

Table 4.1. Characterization of groups in *Erwinia amylovora*.

Groups	Tests that differentiate strains among the groups ^a	Tests that differentiate strains within the groups ^a
<i>Maloideae</i> ^b	Pathogenicity on <i>Rubus</i> , pEA29-PCR, PCR ribotype, rep-PCR fingerprint, <i>hrp</i> gene RFLP, RAPD	Colony morphology PFGE, RAPD, pEA29-PCR and restriction enzyme (RE) type, rep-PCR fingerprint, PCR ribotype
Subgroups		
Differentially virulent	–	Differential host
Streptomycin-resistant	–	Streptomycin phenotype
Austrian	–	pEA29-PCR and RE type
European	–	PFGE
Hokkaido ^c (Japan)	Pathogenicity on <i>Malus</i> , <i>hrp</i> gene RFLP, RAPD, pEA29-PCR	RAPD, <i>hrp</i> gene RFLP
Subgroups		
Hokkaido I and II	–	RAPD, pEA29-PCR
<i>Rubus</i>	Pathogenicity on <i>Malus</i> , <i>hrp</i> gene RFLP, RAPD, pEA29-PCR, rep-PCR fingerprint, PCR ribotype	<i>hrp</i> gene RFLP, RAPD, rep-PCR fingerprint, pEA29-PCR
Subgroups		
<i>Rubus</i> I and II	–	<i>hrp</i> gene RFLP, RAPD, pEA29-PCR

^aAt least some strains are differentiated based on the test mentioned in the table.

^b*Prunus* isolates included with *Maloideae* strains, based on classical characterization of *E. amylovora* (Mohan and Thomson, 1996), were not distinguished from known strain of *E. amylovora*, these have not been characterized yet by RAPD, PFGE, pEA29-PCR and RE type or *hrp* gene RFLP.

^cStrains from Asian pear in Hokkaido, Japan, were treated as a separate group, based on a RAPD relatedness study (Momol *et al.*, 1995; 1997), several biochemical and molecular tests and pattern of pathogenicity to apple cultivars (Beer *et al.*, 1996; Kim *et al.*, 1996). Cited references to tests in some cases may not be the original research papers on the techniques, but rather the use of the technique to differentiate strains: Colony morphology (M.T. Momol and H.S. Aldwinckle, unpublished); Pathogenicity on *Malus* and *Rubus* (*Rubus* strains) (Starr *et al.*, 1951); Differential host (Norelli *et al.*, 1984); *hrp* gene RFLP (Laby and Beer, 1992; Kim *et al.*, 1995, 1996); Streptomycin phenotype (McManus and Jones, 1995); Rep-PCR fingerprint (McManus and Jones, 1995); PCR ribotype (McManus and Jones, 1995); RAPD (Momol *et al.*, 1995; 1997); pEA29-PCR (Kim *et al.*, 1995, 1996); Pathogenicity on *Malus* (Hokkaido strains) (H.S. Aldwinckle, unpublished; Beer *et al.*, 1996); PFGE (Zhang *et al.*, 1996; Zhang and Geider, 1997); PFGE (Merighi, 1996); pEA29-PCR and RE type (Lecomte *et al.*, 1997).

(Lecomte *et al.*, 1997) allowed the grouping of some *Maloideae* strains based on their geographical origins.

Two studies suggested two different geographical origins as the source of *E. amylovora* strains in New Zealand. By fingerprinting strains with PFGE, Zhang and Geider (1997) found that strains from New Zealand had identical patterns to strains from Central Europe. In an earlier fingerprinting study based on rep-PCR analysis, McManus and Jones (1995) suggested that *Maloideae* strains from the USA and New Zealand were very closely related. Based on the results of these studies, it may be that strains of *E. amylovora* have been introduced to New Zealand from two independent sources.

Based on several methods cited in this chapter, *E. amylovora* strains can be classified in three groups: *Maloideae*, *Rubus* and 'Hokkaido'. The main criteria that distinguish these three groups are their specialization toward host and molecular fingerprinting analyses (Table 4.1). In addition, there are subgroups within each group that have been discriminated by molecular and biochemical techniques.

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Migration of *Erwinia amylovora* in Host Plant Tissues

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Introduction

Fire blight, caused by *Erwinia amylovora* (Burrill) Winslow *et al.*, is a devastating bacterial disease of apples and pears and also affects other plants of the *Rosaceae* (see Momol and Aldwinckle, Chapter 4). The economic importance of fire blight is not always easy to appreciate, as it is an erratic disease, but severe outbreaks regularly result in several million US dollars' worth of damage (see van der Zwet and Bonn, Chapter 3). The severity and the huge economic impact of these outbreaks are a consequence of one of the characteristics of *E. amylovora*: its ability to progress internally in host plant tissues. *E. amylovora* enters the host plant through wounds or natural openings, but it preferentially enters through the nectarthodes present in the nectarial cup. Flower infections lead to the loss of the current year's crop. However, on a sensitive host, when the climatic conditions are favourable, the bacterium moves rapidly from the flower to the pedicel and then to the twig, reaching the main branch, and might proceed down the trunk, killing the entire tree. This migration results in the loss of branches or trees, and it might take several years for a severely affected orchard to return to full production. This is when costs associated with fire blight can sky-rocket. This internal progression of *E. amylovora*, as revealed by the evolution of the symptoms, is a characteristic of fire blight that was noted and studied very early on (Jones, 1909; Brooks, 1926; Rosen, 1929, 1936; Pierstorff, 1931).

Few plant-pathogenic bacteria share this ability to migrate in the host tissues. Even when a plant appears totally affected, bacteria are usually localized in some parts of the plants. In the case of wilting caused by *Ralstonia solanacearum*, for example, bacteria invade the vascular tissues and migrate via

the xylem vessels, disrupting the water flow, which leads to the wilting (Hayward, 1995). Consequently, *R. solanacearum* cannot always be isolated from the wilted top part of an affected plant. In contrast, *E. amylovora* is easily isolated from or in front of fire blight symptoms, which include water soaking, wilting and necrosis of the succulent tissues. Therefore, the evolution of the necrosis is the direct consequence of the progression of *E. amylovora* in the plant tissues. *E. amylovora* can be described as inducing an evolutive necrosis; it can migrate inside the plant tissues from the top of the tree down to the rootstock, leaving behind tissues that will rapidly necrose. It is important to note that the wilting associated with fire blight invasion of young shoots and sometimes called 'shepherd's crook' (see Colour Plate Section) is not due to a disruption of the water flow following plugging of the vessels by *E. amylovora* or its exopolysaccharide. It is due to the collapse of the parenchyma. A loss of turgidity of plant cells in tissues recently colonized by *E. amylovora* was already noted by Bachmann in 1913 and later by other laboratories (Huang and Goodman, 1976). Sometimes, strands or drops of exudate can be detected ahead of the symptoms described above. This exudate, which is composed of bacteria embedded in exopolysaccharides, is another characteristic symptom of fire blight. Production of exudate is also a consequence of *E. amylovora* migration in the tissues.

This ability to rapidly invade the host plant has been the subject of a number of studies. However, today there is no consensus on how *E. amylovora* migrates and invades the tissues of a host plant. One of the few facts everybody agrees on is that *E. amylovora* does not produce cell wall-degrading enzymes. No pectolytic, cellulolytic or xylolytic enzyme activities were described in culture media or in cotoneaster tips, apple shoots or immature pear fruits infected by *E. amylovora* (Seemuller and Beer, 1976). This is further supported by the fact that *E. amylovora* cannot macerate potato tubers or carrot slices. *E. amylovora* does not progress in the plant by dissolving the tissues. It is also clear that understanding how *E. amylovora* travels could help to control fire blight and to reduce the cost associated with severe outbreaks. Studies on how *E. amylovora* migrates in the tissues focused first on where *E. amylovora* was in the plant, especially in tissues expressing symptoms. But the situation became more complex after finding *E. amylovora* in symptomless tissues or symptomless plants. This led to the proposition that such latent infections could explain rootstock infections and sudden explosion of fire blight in orchards and nurseries where no fire blight had ever been seen before and away from any obvious sources of inoculum. If migration of *E. amylovora* in host plant tissues can result in the presence of bacteria in symptomless plants, which can later manifest fire blight symptoms, then it is crucial that we understand how *E. amylovora* travels through the tissues. This is particularly true if we want to better control the geographical spread of this disease. In this context, migration of *E. amylovora* also has obvious consequences for quarantine. Clearly, understanding how and where *E. amylovora* migrates in the tissues goes beyond academic interest and has some real practical and economically important outcomes. In this chapter, we shall first

examine some of the tools available to study *E. amylovora* migration in plant tissues. We shall review the results obtained so far and try to explain some of the characteristics of fire blight in light of these results.

A lack of appropriate tools

Trying to understand how *E. amylovora* migrates in plant tissues has been rendered difficult by a lack of appropriate tools. This might explain why, following an early keen interest in this subject at the beginning of the 20th century, few laboratories have recently tried to tackle it. The first dilemma facing scientists who want to understand how *E. amylovora* progresses in the plant is which plant material to use. The most representative plant material is probably a mature apple or pear tree. The most practical material, especially for laboratory experiments, is undoubtedly young plantlets or seedlings. Some experiments would be extremely difficult to conduct with trees, but the development of symptoms in a seedling, where tissues are in a juvenile state, might not allow the extrapolation of the results to mature trees. Experiments have been conducted on trees several years old (Hickey *et al.*, 1999), as well as on 1-week-old seedlings (O'Brien, 1993).

The next dilemma scientists have to confront is how to inoculate the plant material. Inoculation without wounding necessitates the presence of flowers or results in an inconsistent and very low percentage of infection. Artificial inoculation, while giving a consistent and predictably high level of infection, can allow the bacteria to gain access to tissues they might not normally invade. This renders the analysis more difficult and potentially misleading. Finally, there is the problem of detection of the bacteria in the tissues. Early investigators relied on the microscopic examination of the tissues (for example, Bachmann, 1913; Crosse *et al.*, 1972; Eden-Green, 1972) and isolation of the bacteria from the tissues (for example, Lewis and Goodman, 1965; van der Zwet and van Buskirk, 1984; Momol *et al.*, 1998). Then, when the technology became available, they used electron microscopy (for example, Suhayda and Goodman, 1981b) or fluorescent antibodies (for example, Hockenhull, 1979). Recently, new tools, such as amplification of a specific fragment of DNA by PCR, have been developed for *E. amylovora* (Bereswill *et al.*, 1992, 1995; McManus and Jones, 1995; Maes *et al.*, 1996) and used for the detection of *E. amylovora* in symptomless tissues (McManus and Jones, 1995; Maes *et al.*, 1996). The sensitivity and specificity of detection using PCR, which is already excellent, can be further increased using some technical variation of PCR, such as nested PCR (McManus and Jones, 1995). This technique allowed the authors to detect less than one viable cultivable cell of *E. amylovora*. This high sensitivity can be explained by the fact that not all viable *E. amylovora* are cultivable, but also that this technique relies on the presence of a specific DNA fragment rather than the presence of a bacterial cell. Presence of a single DNA fragment is enough to give a positive reaction by PCR. This extraordinary sensitivity is also the main limit and the main

problem of this technique. Detection of *E. amylovora* by PCR does not guarantee that *E. amylovora* cells were present, alive or viable. One has to wonder what the epidemiological significance is of a low population of bacteria that might not be able to multiply.

An additional problem linked with these methods of detection is that they are only a snapshot of a situation that can evolve rapidly. These techniques do not always allow us to infer a causal link between the presence of bacteria in a certain tissue at a certain time and the presence or absence of symptoms observed before tissues were sampled. Symptoms such as production of exudate and necrosis are expressed only in tissues already invaded by *E. amylovora*.

The use of derivatives of *E. amylovora* expressing genes whose product is easily detectable, such as the *lux* operon from *Vibrio fischeri*, which under the right conditions produces light, or the *gfp* genes from *Aequorea victoria*, which produce the green fluorescent protein (GFP), could in theory overcome this problem by allowing detection of bacteria in the tissues without disruption of the tissues. Such derivatives have been constructed and used to inoculate young apple seedlings (Bogs *et al.*, 1998). However, production of light by the *lux* operon occurs only in actively growing bacteria for which sufficient ATP is available. This might not be the case of all cells of *E. amylovora* invading a tissue. Detection of the GFP protein, which is produced constitutively, requires a microscope. This might limit the type of tissues which can be studied easily, since the bacteria might not be easily detected if they are not near the plant surface, thus limiting the use of such derivatives to study colonization of young seedlings.

A link between site of early bacterial multiplication and port of entry

A critical review of the literature published in the 1970s and 1980s clearly shows that the site of bacterial multiplication, or at least early multiplication, is mainly determined by the method of inoculation. Several authors showed that, when *E. amylovora* is applied to a freshly cut leaf, some bacteria are sucked up into the xylem vessels and some of them travel through the xylem vessels of the leaf traces to the main stem (Crosse *et al.*, 1972; Eden-Green, 1972; Hockenull, 1979). Huang and Goodman (1976) found that, when *E. amylovora* is applied directly to an apple petiole, some bacteria move from this petiole to the main stem either by the xylem vessels or by the intercellular spaces of the cortex. Finally, when Suhayda and Goodman (1981b) inoculated apple shoot stems with *E. amylovora*, they observed, in the 48 h following inoculation, an intense bacterial multiplication in the first 10 mm of xylem from the inoculation point. In this experiment, the authors did not report *E. amylovora* in the cortical parenchyma 48 h after inoculation, leading them to conclude that early multiplication was limited to the xylem.

To circumvent problems associated with artificial inoculation of leaves or shoots, Bachmann (1913) and Rosen (1936) studied migration of *E. amylovora*

in tissues after inoculation of pear or apple flowers. When a bacterial suspension is sprayed directly on young flowers, infection occurs readily without the need to cause injury. Both authors concluded that *E. amylovora* was migrating in the intercellular space of the style or nectarial cup (Rosen, 1936) and pedicel (Bachmann, 1913), after entering the plant through the flower. In another attempt to infect plants without causing injury, Bogs *et al.* (1998) placed bacteria on small soaked paper discs directly on to the lower leaf epidermis of young apple leaves. Since this experiment was carried out with a derivative of *E. amylovora* containing the *gfp* gene, the authors were able to follow the infection and the migration of *E. amylovora* in the tissue, using a confocal microscope (Bogs *et al.*, 1998). They concluded that *E. amylovora* could enter plant tissues through broken leaf hairs and then gain access to the xylem (Bogs *et al.*, 1998). Interestingly, almost 70 years earlier, Tullis (1929) also studied the migration of *E. amylovora* in infected apple leaf inoculated without any mechanical injury. He simply sprayed an aqueous suspension of the pathogen on young developing leaves. It has to be noted that the percentage of crab-apple shoots infected in Tullis' experiments varied from 24% to less than 1%. This might reflect the difficulty *E. amylovora* has in entering leaf tissue when no injury is present. No percentage of infection was given by Bogs *et al.* (1998) when using a soaked paper disc to inoculate young apple seedlings. Tullis concluded, just as Heald suggested before him (Heald, 1915), that *E. amylovora* was gaining access to the plant tissues through the stomata. In contrast to Bogs *et al.* (1998), Tullis concluded that, from the point of infection, *E. amylovora* moved downward through the intercellular spaces of the mesophyll along a vein.

From these last two examples, it would seem that how the bacterium migrates in the plant after natural infections is also determined by where *E. amylovora* gained entry in the plant (broken leaf hair or stomata). However, it has to be noted that the pictures presented in support of *E. amylovora* being in the xylem do not rule out that the bacteria were in the intercellular spaces of the mesophyll along the vein. This would be consistent with results from Haber (1928) and Tullis (1929).

***E. amylovora* in diseased tissues**

Following flower infection through the nectarthodes or leaf infection through the stomata, *E. amylovora* must, if only for a short period of time, travel through the intercellular spaces of different tissues. This was actually ascertained by several authors (Bachmann, 1913; Tullis, 1929; Rosen, 1936). This was also found to be the case by Nixon (1927) and after artificial inoculation (Huang and Goodman, 1976). This early intercellular migration results in symptom development. This advance of bacteria in the tissues might be seen as a simple consequence of the increased physical pressure in the intercellular space due to bacterial multiplication (Eden-Green, 1972) or absorption of water by the exopolysaccharide (Schouten, 1989). Such pressure being exerted on all sides

of the intercellular space pushes the bacteria to move, seemingly in all directions, as was described by Haber as early as 1928. However, in this scenario, bacteria are most likely to move according to the path of least resistance. This path might sometimes lead masses of bacteria to the outside of the plant. This could explain the presence of exudate or ooze ahead of any other symptoms. It would also explain why drops of ooze are more easily detected after rain or in a humid environment, when water might suddenly be made available to bacteria, whose masses can suddenly expand following the swelling of exopolysaccharides absorbing this water.

Multiplication of *E. amylovora* in the intercellular spaces results in symptom development and can explain migration of the bacteria in the tissues. This, however, does not exclude a priori that under some conditions multiplication and migration of *E. amylovora* in the vascular tissues is also associated with the production of symptoms in another part of the plant, as suggested in leaf infections studied by Crosse *et al.* (1972).

One laboratory concluded that *E. amylovora* invaded the phloem, resulting in an upward movement of the bacteria (Lewis and Goodman, 1965; Gowda and Goodman, 1970). Such a conclusion was reached after the authors were able to stop the progression of the disease by removing a 3 cm segment of tissue from the outer bark down to the xylem of 1-year-old apple trees. This hypothesis has never been confirmed by more meticulous and in-depth experiments and the observed results are equally consistent with migration of bacteria in the cortical parenchyma. This same laboratory also concluded that *E. amylovora* grows in the xylem and plugs the vessels, leading to the wilting associated with fire blight (Goodman and White, 1981; Suhayda and Goodman, 1981a, b). This hypothesis not only cannot explain the other symptoms associated with fire blight but is also in sharp contrast with the results of earlier studies on apple and hawthorn, which associate fire blight symptoms with bacterial invasion of the cortical parenchyma (Bachmann, 1913; Rosen, 1936; Eden-Green, 1972; Hockenhull, 1979). Eden-Green (1972) came to such a conclusion after careful histological examinations of apple stem tissue infected by *E. amylovora* over a period of 7 days. From the first centimetre to as far as 27 cm from the point of inoculation, massive bacterial multiplication occurred almost exclusively in the cortical parenchyma. Exceptionally, *E. amylovora* was also detected in the xylem vessels of diseased plants, but this was attributed to accidental introduction of the bacteria into this tissue during inoculation.

***E. amylovora* in symptomless tissues**

Several authors, like Eden-Green (1972), detected *E. amylovora* in the xylem vessels of symptomless tissue or symptomless plants (Rosen, 1929; Shaw, 1934), but this was not associated with symptom development. Bacteria isolated ahead of tissues expressing symptoms are virulent (Keil and van der Zwet, 1972; Aldwinckle and Preczewski, 1976). Eden-Green (1972) noted that inoculation

involving damage that permitted introduction of bacteria into vascular tissues resulted in the persistence of *E. amylovora* in xylem vessels of symptomless tissues of the most resistant apple cultivars. Restriction of the bacteria to the vessels might not allow sufficient multiplication to give rise to expression of symptoms. For much of the year, the xylem vessels bring mostly water and salts from the roots to the leaves and might not contain all the elements necessary for high-rate multiplication of *E. amylovora*. This would explain why persistence of *E. amylovora* in symptomless tissues seems to be limited (Gowda and Goodman, 1970; Ge and van der Zwet, 1996; Momol *et al.*, 1998), although a low number of *E. amylovora* cells seem to be able to survive and move in symptomless plants (Keil and van der Zwet, 1972; Momol *et al.*, 1998). Using the powerful nested PCR tool they developed, McManus and Jones (1995) were able to detect *E. amylovora* from healthy-looking trees from an orchard in which no fire blight symptoms were found that year and in which fire blight had been rare previously. This suggests that only a few bacteria are able to survive for long periods of time in symptomless plants.

It is tempting to speculate that, when *E. amylovora* is in the xylem vessels, it is able to move throughout the plant and to survive, at least for 1 year, but is not able to cause fire blight symptoms, which seem to be associated with the presence of *E. amylovora* in the intercellular space of the cortical parenchyma. Initial attempts to induce symptoms of fire blight from symptomless trees harbouring *E. amylovora* failed (Keil and van der Zwet, 1972). However, if *E. amylovora* could under special conditions escape the containment of the xylem vessels, it could induce fire blight. As noted below, seasonal effects deserve special consideration here. Physiological changes in the host, notably elevated concentrations of certain amino acids in the xylem sap as dormancy is broken in the spring, as well as rising ambient temperatures, may permit rapid bacterial multiplication in the xylem and subsequent release via weakened or damaged tissues such as those at the margins of previously dormant cankers.

Epidemiological considerations

As mentioned in the introduction, understanding where *E. amylovora* multiplies and how it migrates in the plant might help to explain some epidemiological characteristics of fire blight, such as rootstock infection and sudden outbreaks of fire blight in the absence of any obvious source of inoculum.

Rootstock infections have been reported from France (Huberdeau *et al.*, 1992) and from the USA, especially in high-density apple orchards planted on M.9 or M.26 rootstocks (Momol *et al.*, 1998, 1999; Steiner, Chapter 17). The symptoms associated with rootstock infections are different from the 'classic' symptoms of fire blight described in the introduction. In spring, rootstock infections are revealed by a delayed bud break, followed by poor growth or even the death of the tree. The sudden death of a tree in mid-season can also be due to rootstock infection. Most often, however, it is during autumn that symptoms are

the most dramatic. Leaves get an early red colour and cling to the tree. Losses due to rootstock infection can be severe: 60–80% of the trees over a 2-year period (Steiner, Chapter 17). Up to 40% of trees were showing symptoms of rootstock infection in some parts of France in 1991 (Huberdeau *et al.*, 1992). In one year, 10% of apple trees in an orchard of 'Braeburn' grafted on M.26 rootstock in New York state were lost due to rootstock infection (Momol *et al.*, 1999).

Infection of suckers or water sprouts could clearly lead to rootstock infection. However, rootstock infections have been observed in the absence of any infected sucker (Huberdeau *et al.*, 1992; Momol *et al.*, 1998). In these cases, it is tempting to speculate that *E. amylovora* invaded the rootstock tissues after internal migration. *E. amylovora* could have entered the plant tissues following blossom or shoot infection, got into xylem vessels and migrated into the rootstock. Momol *et al.* (1998) showed that, after artificial inoculation, *E. amylovora* could move from a scion to the rootstock through symptomless tissues. However, in 1991, rootstock infection in France was preceded by a severe fire blight outbreak the previous season (Huberdeau *et al.*, 1992), and no symptoms are associated with the presence of *E. amylovora* in the xylem until it can escape from the vessels and get into the cortical parenchyma, where conditions allow for rapid bacterial multiplication and symptom development. In this scenario, rootstock infection would occur because the bacteria are at the end of their migration localized only in the xylem vessels of the rootstock, and therefore escape from those tissues to give infection, or because for some reason such as physiological differences between the rootstock and the rest of the plant it is easier for the bacteria to invade the cortical parenchyma when in the xylem vessels of the rootstock rather than when in the xylem vessel of another part of the tree.

Sudden and unexpected outbreaks of fire blight in areas where there is no inoculum available (Thomson, Chapter 2) can be explained in a similar fashion. Following infection, *E. amylovora* gets trapped in the xylem vessels until it can escape into the cortical parenchyma and lead to fire blight symptoms. In this case, two parts of the plant might allow *E. amylovora* to escape, either the rootstock or the juvenile tissues present at the shoot tips.

Haber (1928) was the first to observe that in apple leaves, even if *E. amylovora* was inoculated through a vein, it could spread easily in the surrounding parenchyma. Then Shaw (1934) indicated that *E. amylovora* could get out of xylem vessels especially at the tip of a shoot. More recently, Bogs *et al.* (1998) reported that, following inoculation of cut or wounded leaves of apple seedlings, *E. amylovora* could invade the leaf parenchyma from the xylem vessels of leaf veins. This ability of endophytic *E. amylovora* to cause symptoms at a later date has some important consequences for the epidemiology and control of dispersal of the disease.

However, there are several important questions which are still unanswered, such as: How and why can *E. amylovora* sometimes escape xylem containment? If there is a need for a trigger to allow invasion of the cortical parenchyma, what is this/these triggers? Is *E. amylovora* contained in the xylem simply by lack of

nutrients to support active growth? Answers to these questions would help us to understand some of the very frustrating characteristics of fire blight, such as sudden outbreaks in the absence of inoculum, and would, perhaps, help us to control the geographical spread of the disease.

Conclusion/summary

Migration of *E. amylovora* in host plant tissues has been a controversial subject and one that is technically difficult to address. It is likely to be influenced by the mode of entry into the plant as well as the type and physiological susceptibility (age, phenology, variety and species) of the tissue invaded. Few studies have considered these factors in a systematic manner and the literature is thus replete with confusing observations and sometimes conflicting conclusions.

It seems clear that multiplication of *E. amylovora* in the intercellular space of the cortical parenchyma can result in migration of the bacteria in the host plant tissues, and that this can account for the series of symptoms characteristic of fire blight in the current seasons's growth; including necrosis, loss of mechanical strength resulting in crooking or 'wilting', and the ready emergence of bacterial exudate as ooze or strands. As leaves, flowers and actively growing shoot tips are the tissues most vulnerable to natural infection it is likely that this initial mode of migration is common. Where such tissues cease to be susceptible, through age or physiological status of the host or environmental effects on the bacterium, invasion of parenchymatous tissues may become limited, with the production of cankers having more or less defined margins. This process probably involves active host resistance mechanisms which, in some cases, clearly cease to be effective when such cankers become active in the spring. Are such sources of infection the result of renewed invasion by bacteria surviving in the bark or alternatively, of endogenous invasion from within the woody tissues?

Sometimes *E. amylovora* gets sucked into the xylem vessels. Symptoms are not usually observed whilst bacteria are confined to these tissues, but the pathogen seems able to migrate rapidly, and considerably beyond the point of initial entry, and to multiply at levels sufficient to ensure survival for at least one season while being contained in the xylem vessels. Invasion of xylem tissues, in advance of or remote from colonization of parenchyma in the bark, may account for the rapid spread and subsequent perennation of the pathogen. Under conditions that as yet are incompletely understood, it would appear that *E. amylovora* can escape from the xylem vessels and invade the cortical parenchyma inducing typical fire blight symptoms, or atypical symptoms when only the rootstock gets infected. As first suggested more than 100 years ago by Waite (1896), bacterial multiplication in the xylem might be triggered by seasonal changes in the composition of xylem sap, and subsequent invasion could account for, or contribute to, the re-activation of overwintering cankers. How, and under what conditions, *E. amylovora* is able to break out from the containment of the xylem vessels is still unknown.

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The Pathogen



Erwinia amylovora: General Characteristics, Biochemistry and Serology

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Introduction

Many general characteristics of a bacterium can be assumed by its name. Nomenclatures are based on studies that provide clear and reliable placement of bacteria into groups. In this chapter, we shall present the description of the species *Erwinia amylovora* as given by the taxonomists. Such a description is necessarily general, gathering the common traits of individuals of the species. It provides a means to separate this species from other groups of organisms and gives a minimal picture of what is generally recognized, by microbiologists and phytopathologists, as the species *E. amylovora*. Then, additional information on the cultural and morphological characteristics, physiology and nutrition, serology and phage sensitivity of *E. amylovora* will be presented.

Present taxonomic description

It is not intended to review here the long history of changes in nomenclature and classification of plant-pathogenic bacteria. But it seems important to give the full 'official' description of the species. Such a description is provided by Lelliott and Dickey (1984), in *Bergey's Manual of Systematic Bacteriology*, 8th edition (Krieg and Holt, 1984), and the essential characters are summarized in *Bergey's Manual of Determinative Bacteriology*, 9th edition (Holt *et al.*, 1994).

The genus *Erwinia*

The genus *Erwinia* has been reviewed several times (Starr and Chatterjee, 1972; Starr, 1981; Slade and Tiffin, 1984; Perombelon, 1992, 1994). This genus was initially created to place together the *Enterobacteriaceae* (Gram-negative, motile, aerobic-facultative anaerobic, non-sporulated) which are ecologically associated with plants (Brenner, 1984). *E. amylovora* is the type species of this genus. This original definition shows some obvious limitations and resulted in a somewhat artificial grouping of microorganisms that are not necessarily genetically or phylogenetically closely related. This is why proposals to break this genus into several pre-existing or new genera among *Enterobacteriaceae* are recurrent (Starr and Chatterjee, 1972; Starr, 1981). Such proposals may be supported by studies on DNA relatedness among species of this genus (Gardner and Kado, 1972).

A grouping within erwinias has been accepted, following the work of Dye (1968, 1969a, b, c). The '*amylovora*' group, the '*carotovora*' group and the '*herbicola*' group are expressions still in use among plant pathologists, to designate, respectively, the wilt and necrosis pathogens the soft rot pathogens and the saprophytic bacteria (usually yellow), sharing general features of the *Enterobacteriaceae*. Analysis of DNA hybridization showed a significant degree of relatedness within the '*amylovora*' group (Brenner *et al.*, 1974). Simple biochemical tests, such as glucose fermentation, nitrate reduction, nitrate respiration, nucleoside phosphotransferase and pectolytic enzyme production, allowed the differentiation of these groups in the laboratory (Komagata *et al.*, 1968). However, more comprehensive numerical taxonomy studies (Dye, 1981; Mergaert *et al.*, 1984; Verdonck *et al.*, 1987) did not really support this view; this grouping is no longer accepted at the taxonomic level. The genus *Erwinia* is now presented as a single group of 15 species (Lelliott and Dickey, 1984), more recently extended to 17 (Holt *et al.*, 1994), which include, in fact, species formerly placed in the '*amylovora*' group and the '*carotovora*' group and some pathogenic species from the '*herbicola*' group. It is recognized, though not commonly accepted, that the bacteria from the former '*carotovora*' group could be taxonomically better placed in another genus, such as *Pectobacterium* (Lelliott and Dickey, 1984). Saprophytic bacteria from the former '*herbicola*' group are now placed in *Enterobacter agglomerans* (Ewing and Fife, 1972) (syn. *Pantoea agglomerans*) (Holt *et al.*, 1994). Recently, a comprehensive study of the sequence of 16S rDNA of 29 strains of *Erwinia*, *Pantoea* and *Enterobacter* (Hauben *et al.*, 1998), allowed the grouping of species of *Erwinia* strains into three phylogenetic groups: cluster I is considered to represent the true *Erwinia* and contains *E. amylovora*. Cluster II includes the pectinolytic strains of the former '*carotovora*' group. The name *Pectobacterium* is indeed proposed as the genus name for these bacteria. In addition, a cluster III contains a number of other plant pathogenic erwinias, under the proposed name of *Brenneria*. The formerly described *Pantoea* genus forms a cluster IV, related to *Erwinia*, but distinct.

The species *E. amylovora*

The description of the species, according to Lelliott and Dickey (1984), is currently as follows:

Erwinia amylovora (Burrill 1882) Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1920 (*Micrococcus amylovorus*, Burrill 1882). Colonies on 5% sucrose nutrient agar are typically white, domed, shiny, mucoid (levan type) with radial striations and a dense flocculent centre or central ring after 2 or 3 days at 27°C. Non-levan forms are rarely isolated. Agglutination with *E. amylovora* anti-serum is the most rapid and accurate method of determination (Lelliott, 1967); the species is serologically homogeneous and has few agglutinogens in common with related species or with the saprophytes found in diseased material. Causes a necrotic disease (fire blight) of most species of the *Pomoideae* and of some species in other subfamilies of the *Rosaceae*. A *forma specialis* has been described from raspberry (*Rubus idaeus*) by Starr *et al.* (1951). The mol% G + C of the DNA of seven strains ranges from 53.6 to 54.1 (buoyant density).

This description has been supplemented by Rijckaert (1994), cited in Hauben *et al.* (1998) with the following data:

there is no anaerobic growth and strains hydrolyse esculin. All strains produce acid from sorbitol. Strains grow on melibiose and sorbitol as carbon sources. Strains can use isoleucine, methionine and threonine as nitrogen sources. Strains are sensitive to furazolidone.

A biochemical characterization allowing a distinction between species of *Erwinia* is proposed in Holt *et al.* (1994). It stresses that differentiation of *E. amylovora* from other species of *Erwinia* relies only on seven positive cultural physiological characteristics, out of the 28 used for the genus (Table 6.1). These characteristics are: motility, weak anaerobic growth, mucoid growth, reducing substance from sucrose, production of acetoin (in shaken culture) and liquefaction of gelatin. As far as acid production from organic compounds is concerned (Table 6.2), out of 24 compounds tested, only two consistently yielded a positive response (ribose, trehalose), while two more were 'frequently' positive (arabinose, sorbitol). As sources of carbon and energy (Table 6.3), *E. amylovora* utilizes the following organic sources: citrate, formate and lactate, but not tartrate, galacturonate or malonate. For growth in minimal medium, *E. amylovora* exhibits an absolute requirement for nicotinic acid.

This brief description is necessarily a picture of great homogeneity. Such a homogeneity within the species *E. amylovora* has been recognized for a long time (Ark, 1937; Hildebrand, 1954), and was confirmed when populations of isolates were studied after introduction of the disease in Europe (Billing *et al.*, 1961; Paulin and Samson, 1973), even when a large number of isolates was tested (Vantomme *et al.*, 1982, 1986; Verdonck *et al.*, 1987).

It is likely that recent development of molecular techniques, which allow comparisons of the genomic organization of strains, will provide a more complete information on isolates, and some differences between strains of diverse

Table 6.1. Cultural, physiological and biochemical characteristics^a of *Erwinia* species (from Holt *et al.*, 1994).

Test	<i>E. amylovora</i>	<i>E. ananas</i>	<i>E. carotovora</i>	<i>E. chrysanthemi</i>	<i>E. cypripedii</i>	<i>E. malloivora</i>	<i>E. nigrifluens</i>	<i>E. quercina</i>	<i>E. rhapontici</i>	<i>E. rubrifaciens</i>	<i>E. salicis</i>	<i>E. stewartii</i>	<i>E. tracheiphila</i>	<i>E. uredoovora</i>
Motility	+	+	+	+	+	+	+	+	+	+	+	—	+	+
Anaerobic growth	W	+	+	+	+	+	+	+	+	+	W	+	W	+
Growth factors required	+	—	—	—	—	+	—	+	—	—	—	—	+	—
Pink diffusible pigment	—	—	—	—	—	—	—	—	+	+	—	—	—	—
Blue pigment	—	—	—	d	—	—	—	—	—	—	—	—	—	—
Yellow pigment	—	+	—	—	—	—	—	—	—	—	—	+	—	+
Mucoid growth	+	+	d	d	d	+	—	+	+	+	+	+	—	—
Sytoplasmata	—	—	—	—	—	—	—	—	—	—	—	—	—	d
Growth at 36°C	—	+	d	+	+	—	+	+	d	+	—	d	—	+
H ₂ S from cysteine	—	d	+	+	+	—	+	+	+	+	+	—	+	—
Reducing substances from sucrose	+	+	d	—	—	+	—	+	d	—	+	d	d	+
Acetoin	+	+	+	+	—	+	+	+	+	—	+	—	d	+
Urease	—	—	—	—	—	—	+	—	—	—	—	—	—	—
Pectate degradation	—	—	+	+	—	—	—	—	—	+	+	—	—	—
Gluconate oxidation	—	—	—	—	+	—	—	—	d	—	—	—	—	—
Gas from D-glucose	—	—	d	+	+	—	—	—	—	—	—	—	—	—
Casein hydrolysis	—	—	d	d	—	—	—	—	—	—	—	—	—	—
Growth in KCN broth	—	—	d	d	+	—	—	—	+	—	—	—	—	—
Cottonseed oil hydrolysis	—	—	d	d	+	—	—	—	d	—	—	—	—	—
Gelatin liquefaction	+	+	+	+	—	—	—	—	—	—	—	—	—	+
Phenylalanine deaminase	—	—	—	—	+	—	—	—	—	—	—	—	—	—
Indole	—	+	—	+	—	—	—	—	—	—	—	—	—	+
Nitrate reduction	—	—	+	+	+	—	—	—	+	—	—	—	—	+
Growth in 5% NaCl	—	+	+	d	+	—	—	—	+	—	—	+	—	+
Deoxyribonuclease	—	—	—	—	—	—	—	—	—	—	—	—	—	+
Phosphatase	—	—	—	+	d	—	—	—	d	—	—	—	—	—
Lecithinase	—	—	—	+	—	—	—	—	—	—	—	—	—	—
Sensitivity to erythromycin (15 µg per disc)	—	—	—	+	+	—	—	—	+	—	—	—	—	—

^a See *Bergey's Manual of Systematic Bacteriology* for methods (Krieg and Holt, 1984).

Symbols: +, 80% or more positive; —, 20% or less positive; d, 21–79% positive; W, weak growth; blank spaces, insufficient or no data.

origins (geographical, host plants) have already been described (McManus and Jones, 1995; Beer *et al.*, 1996; Kim *et al.*, 1996; Momol *et al.*, 1997; Momol and Aldwinckle, Chapter 4). In this respect, it can be of interest to note that a bacterium-causing necrosis, resembling symptoms of fire blight on Asian pear tree

Table 6.2. Acid production from organic compounds^a by *Erwinia* species (from Holt *et al.*, 1994).

Compounds	<i>E. amylovora</i>	<i>E. ananas</i>	<i>E. carotovora</i>	<i>E. chrysanthemi</i>	<i>E. cypripedii</i>	<i>E. mallotivora</i>	<i>E. nigrifluens</i>	<i>E. quercina</i>	<i>E. thapontici</i>	<i>E. rubrifaciens</i>	<i>E. salicis</i>	<i>E. stewartii</i>	<i>E. tracheiphila</i>	<i>E. uredovora</i>
D-Adonitol	—	—	—	—	—	—	—	—	—	—	—	—	—	+
L-Arabinose	d	+	+	+	+	—	+	—	+	+	—	+	—	+
Cellobiose	—	+	+	+	+	(+)	—	—	+	—	—	—	—	+
Dextrin	—	—	—	—	—	—	—	—	—	—	—	—	—	+
Dulcitol	—	—	—	—	—	—	—	—	d	—	—	—	—	—
Esculin	—	d	+	+	+	—	+	+	+	—	+	—	—	d
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-CH ₂ -D-glucoside	—	—	d	—	—	—	—	+	d	+	—	—	—	—
Glycerol	—	+	d	+	d	(+)	+	+	+	d	d	—	—	+
Myo-inositol	—	+	d	d	+	—	+	—	+	—	+	—	—	+
Inulin	—	d	—	d	—	—	—	—	+	—	—	d	—	+
Lactose	—	+	+	d	—	—	—	—	+	—	—	+	—	+
Maltose	—	+	d	—	+	—	—	—	+	—	—	—	—	+
D-Mannitol	—	+	+	+	+	+	+	+	+	+	+	+	—	+
D-Mannose	—	+	+	+	+	+	+	+	+	+	+	+	—	+
Melezitose	—	—	—	—	—	—	—	—	d	—	—	—	—	+
Melibiose	—	+	+	+	+	—	+	—	+	—	+	+	—	+
Raffinose	—	+	+	+	—	—	+	—	+	—	+	+	—	+
L-Rhamnose	—	d	+	+	+	—	+	—	+	—	—	—	—	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	—	+
Salicin	—	+	+	+	+	—	+	+	+	—	+	—	—	d
D-Sorbitol	d	+	+	+	+	—	+	+	+	+	+	+	—	+
Starch	—	+	—	—	—	—	—	—	+	—	—	—	—	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	—	+	+	+	—	+	—	—	+	—	+
D-Xylose	—	+	+	+	+	+	+	—	d	—	—	+	—	+

^a After 7 days' growth at 27°C in unshaken aqueous solution of 1% organic compound, 1% peptone with bromocresol purple as an indicator.

Symbols: (+), delayed positive reaction; +, 80% or more positive; —, 20% or less positive; d, 21–79% positive.

(*Pyrus pyriolia*), in Korea has been described as a new species, *Erwinia pyrifoliae*, clearly distinct from *E. amylovora* (Kim *et al.*, 1999) on genotypic and phenotypic basis.

A review on *E. amylovora*, orientated mainly towards aspects of pathogenesis and host specificity, has recently been published (Vanneste, 1995).

Table 6.3. Utilization of some organic compounds as a source of carbon and energy for *Erwinia* species^a (from Holt *et al.*, 1994).

Compounds	<i>E. amylovora</i>	<i>E. ananas</i>	<i>E. carotovora</i>	<i>E. chrysanthemi</i>	<i>E. cyripedii</i>	<i>E. mallovora</i>	<i>E. nigrifluens</i>	<i>E. quercina</i>	<i>E. rhapontici</i>	<i>E. rubrifaciens</i>	<i>E. salicis</i>	<i>E. stewartii</i>	<i>E. tracheiphila</i>	<i>E. uredovora</i>
Citrate	+	+	+	+	+	+	—	+	+	+	—	+	d	+
Formate	+	+	+	+	+	—	+	+	+	+	—	+	d	+
Lactate	+	+	+	+	+	—	+	+	+	+	—	+	—	+
Tartrate	—	+	—	d	+	—	+	—	d	+	—	+	—	+
Galacturonate	—	d	d	d	+	—	—	—	d	—	—	—	—	—
Malonate	—	—	—	+	d	—	—	—	+	—	—	—	—	—

^a In 21 days at 27°C on OY medium (NH₄H₂PO₄, 0.5 g; K₂HPO₄, 0.5 g; MgSO₄·7H₂O, 0.2 g; NaCl, 5 g; yeast extract, 0.08%; water, 1 l).

Symbols: +, 80% or more positive; —, 20% or less positive; d, 21–79% positive.

Table 6.4. Colony characteristics of *Erwinia amylovora* on particular media.

Medium	Medium type ^a	Colony morphology and colour	Reference
SNA	D	Domed, circular, mucoid	Billing <i>et al.</i> (1961)
D 3	S	Clear red coloration of the medium	Kado and Heskett (1970)
MS	S	Red to orange colours	Miller and Schroth (1972)
King B	D	White, circular, mucoid	Paulin and Samson (1973)
CG	S	Typical craters on surface of colonies	Crosse and Goodman (1973)
TTN	S	Chalky white, entire, smooth glossy surface, doughnut-shaped	Ritchie and Klos (1978)
CCT	S	Smooth, large, pulvinate, light blue opalescent, with craters	Ishimaru and Klos (1984)
MM ₂ Cu	D	Yellow, circular, smooth	Bereswill <i>et al.</i> (1997)

^a D, diagnostic medium; S, selective medium.

Cultural and morphological characteristics

Colony morphology – specific media

Colony morphology depends strongly upon the media and growth conditions. Characteristic features of colonies on media specially used for diagnosis of *E. amylovora* are described in Table 6.4. Selective media were developed to allow an easier isolation of *E. amylovora*. Some also gave a somewhat typical aspect to *E. amylovora* colonies, increasing the selectivity of the medium.

The medium that seems to be most commonly used among phytopathologists for isolation of *E. amylovora* is CCT (Ishimaru and Klos, 1984). This

medium has a good level of selectivity. It is composed of sucrose and sorbitol as carbon sources, and of the following inhibitors: tergitol anionic, thallium nitrate, cycloheximide and crystal violet. Representatives of *Erwinia herbicola*, often associated with *E. amylovora* in plant lesions (Billing and Baker, 1963) and certain *Pseudomonas* spp. commonly found on the plant surface, grow on this medium, but their colonies show a distinct morphology.

It has often been noted (Billing *et al.*, 1960; Paulin and Samson, 1973) that on certain media typical and atypical colonies could be obtained from the same isolate, each type being able to give rise to the other type. Such a diversity may be obtained from direct isolation from lesions, as well as from plating a bacterial suspension. These morphological differences are not linked to any known difference in physiology or pathogenicity (Paulin and Samson, 1973).

Cell morphology

Cells of *E. amylovora* are Gram-negative rods of about $0.3\ \mu\text{m} \times 1\text{--}3\ \mu\text{m}$ in size. Size of cells may vary according to growth conditions and techniques of observation (for a review, see van der Zwet and Keil, 1979). After growth on a suitable medium, a variable number of cells are surrounded by a capsule, visible under the microscope, which can be thick or thin. Some cultures are composed entirely of non-capsulated cells (see below), but most of the cultures show a mixture of capsulated and non-capsulated cells (Bennett and Billing, 1978).

Some authors looking for cellular differences between virulent and non-virulent isolates of the pathogen described differences in size of bacterial cells. Two types of cells were described: normal cells ($1.0\text{--}2.5\ \mu\text{m} \times 0.8\text{--}1.2\ \mu\text{m}$) and filamentous cells ($7.0\text{--}35.0\ \mu\text{m} \times 0.8\text{--}1.2\ \mu\text{m}$), the latter being associated with a small colony type on culture medium. The filamentous forms are able to produce 'minicells' (cell wall and cytoplasmic membrane, with no nuclear material). No differences in pathogenicity were actually found between 'normal' and 'filamentous' cell populations (Voros and Goodman, 1965; Huang and Goodman, 1970).

Cell envelopes

Characteristics and ultrastructure

Cell envelopes of *E. amylovora* show an unusual level of susceptibility to a low concentration of surfactant agents (novobiocine, desoxycholate, sodium dodecylsulphate) (Chatterjee *et al.*, 1977). Simultaneously, a spontaneous release of enzymes from the periplasmic space to the external medium suggests some defect in the outer membrane of the bacteria, and a relation with pathogenicity was proposed. The observation of Goodman (1983), who indicated a very short survival time of suspensions of *E. amylovora* in distilled water, may be connected with this characteristic of the outer membrane.

Nevertheless, little original information has been obtained from ultra-structure observation of cell envelopes of *E. amylovora* (Voros and Goodman, 1965; Huang and Goodman, 1970). Even with the use of special techniques (freeze-fracture), no specific traits of bacterial envelopes were described (Gibbins *et al.*, 1976). One work showed the presence of unusual small evaginations in the outer membrane of *E. amylovora* cultures (Laurent *et al.*, 1987). These were present in both virulent and non-virulent strains, and no interpretation for these structures could be provided.

Fatty acids

The lipid composition of cell envelopes, which has been proposed as a suitable criterion for bacterial taxonomy, was studied by gas-liquid chromatography (Casano *et al.*, 1988; van der Zwet and Wells, 1993; Wells *et al.*, 1994). For *E. amylovora*, as for other bacteria, the fatty acid profile may depend to a certain extent on the growth conditions, especially the age of the culture and the composition of the growth medium (Casano *et al.*, 1988). Nevertheless, standard conditions of growth allowed the characterization of a typical profile (Box 6.1), which has been incorporated in a library of profiles, for the identification of *E. amylovora*. This profile was slightly different for *E. amylovora* isolated from *Rubus*, but remained fairly constant for other strains of *E. amylovora*. It was noted that streptomycin variants could be distinguished from wild-type *E. amylovora*, because of a lower percentage of cyclic acids (van der Zwet and Wells, 1993). Other saprophytic *Erwinia* are said to produce profiles that are different, as do other bacterial species, even if they are taxonomically close to *E. amylovora* (Wells *et al.*, 1994). The fatty acid profile has frequently been used as a tool to confirm identification of *E. amylovora* after new introductions, as in Egypt, Bulgaria, Yugoslavia (van der Zwet and Wells, 1993) and Austria (Keck *et al.*, 1997).

Surface receptors

Since the outer membrane of Gram-negative bacteria constitutes an interface between the cell and the external medium, the role of receptors present on the outer membrane received a lot of interest. The composition and structure of

Box 6.1. Fatty acid profile library for *E. amylovora* (143 strains) (from van der Zwet and Wells, 1993).

Unsaturated acids	43%
Saturated straight chains	41%
Hydroxy-substituted acids	7%
Cyclic acids	3%
Saturated branched chains	1%
Unsaturated branched chains	4%

lipopolysaccharides (LPS), which are usually the sites for specific recognition from external factors (O antigen), have been precisely determined (Ray *et al.*, 1986). The lipid fraction was composed of glucosamine, phosphate and three fatty acids (12:0, 14:0 and 3-OH-14:0), which are common to *Enterobacteriaceae*. The carbohydrate fraction was composed of:

- a short side-chain of three neutral sugars: fucose, glucose and galactose;
- a core containing oligosaccharides (heptose, glucose and uronic acid) – which is unusual for *Enterobacteriaceae* – amino compounds and 3-desoxy-2-octulosonic acid (KDO);
- a low-molecular-fraction (KDO, amino compounds and phosphates).

In addition, it was found that these LPS have some common features with *Rhizobium* and, perhaps, with *Erwinia stewartii* and *Erwinia carotovora* (Ray *et al.*, 1986). Small variations were found between virulent and non-virulent strains, and the absence of side-chain for some phage-resistant mutants was noted. The most probable structure of the side-chain of LPS of one 'typical' strain was determined by Ray *et al.* (1987) (Fig. 6.1). It is original in several ways: fucose is in the D configuration, which has also been described for other plant pathogens, but it contains glucofuranose, which has not been reported for other LPS. Glucose and galactose are present, as in the composition of capsular exopolysaccharide (EPS), but their linkage is likely to be different.

The isolation from apple of a factor that agglutinates cells of *E. amylovora* (Romeiro *et al.*, 1981a) resulted in research for a specific receptor on the cell surface of *E. amylovora*. This receptor was supposed to be linked to the LPS. It was found that the agglutinating factor was not linked to the side-chain, but that it was unexpectedly linked to the core of the LPS. Therefore, one could expect that some basis for specificity may lie in the core of the LPS (Romeiro *et al.*, 1981b).

Capsule

The presence of a layer of polysaccharides surrounding the cell, or capsule for *E. amylovora*, has long been noted (Billing, 1960), as well as its role in pathogenicity (Bennett and Billing, 1978, 1980; Ayers *et al.*, 1979). This capsule is

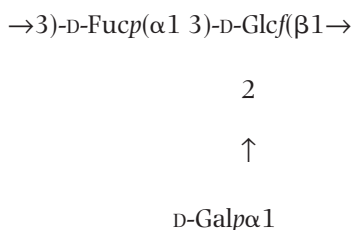


Fig. 6.1. Structure of the side-chain of LPS from *E. amylovora* (from Ray *et al.*, 1987).

composed of galactose, glucose, mannose and uronic acid. A specific enzyme, produced by *E. amylovora*, a galactosyl-transferase presumed to allow the synthesis of EPS during bacterial multiplication within plant tissue, has been isolated (Huang, 1979). Another (extracellular) polysaccharide, a polyfructose called levan, is produced by *E. amylovora*. The role of these polysaccharides will be further described, as well as the genetic, biochemical and pathogenic aspects of the capsule (Geider, Chapter 7).

Physiology and nutrition

Although some studies on the physiology and nutrition of *E. amylovora* were aimed at elucidating the metabolism of *E. amylovora*, most of these studies compared virulent and non-virulent strains, in order to provide a better understanding of the pathogenicity and the ecology of this bacterium. In addition, most data presently available on the nutrition of this bacterium are derived from standard laboratory biochemical tests performed for determination purposes. This information may have little to do with the real metabolism of the bacteria under natural conditions.

Temperature for growth

The relation between temperature and growth of the bacteria in complex liquid media was established by Billing (1974). It showed a linear relationship between doubling rate and temperature between 9°C and 18°C. A sharp change was noticed in the growth rate at 18°C: an increase of 10°C from 18°C induced a moderate decrease in doubling time (from 2.1 h to 1.3 h), while a decrease of 10°C from 18°C induced a high increase of doubling time (from 21 h to 14 h). Therefore this value of 18°C was stressed as probably being significant in the epidemiology of the disease. Interestingly, in the same study, similar values for the relation between growth and temperature were found for *E. amylovora* inoculated into growing apple shoots.

E. amylovora is capable of growth between 3–5°C and 37°C. The optimal temperature is 25–27°C (Billing *et al.*, 1961). Capacity for growth at 34°C was retained as an original feature of *E. amylovora* by Vantomme *et al.* (1986). The resistance of bacterial cells to high temperature was studied, in an attempt to propose a technique to free plant material of internal contaminants by heat treatment (Aldwinckle and Gustafson, 1993; Keck *et al.*, 1995). It was found that a temperature of 45°C for 70 min or 50°C for 50 min was enough to destroy pure cultures of the bacteria. Some variations were noted between strains (Keck *et al.*, 1995).

Fermentative metabolism

E. amylovora is a facultative anaerobe. It produces acid but no gas from glucose, either aerobically or anaerobically. It is one of the three *Erwinia* species, along with *E. salicis* and *E. tracheiphila*, quoted as being weakly fermentative (Holt *et al.*, 1994). All other *Erwinia* are fermentative, while all other plant pathogens, Gram-positive or Gram-negative, are strictly oxidative.

The metabolism of glucose, in connection with oxygen availability in mineral-defined medium, has been studied in a chemostat under precise growth conditions (Farago and Gibbins, 1975). Growth of *E. amylovora* was limited by glucose concentration for dissolved oxygen tension (DOT) exceeding 6 mmHg, while it was limited by oxygen availability when DOT was less than 4 mmHg. As DOT decreased, acid production increased. These transitions from glucose to oxygen limitations were accompanied by sharp changes in specific enzymatic activities (e.g. succinate oxidase, D-glyceraldehyde, 3-phosphate dehydrogenase, D-fructose-1,6-diphosphate aldolase and malate-dehydrogenase). As mentioned by Farago and Gibbins (1975), this shows a high capacity of *E. amylovora* to respond to change in the nutritional status of the environment. A role for this capacity in its behaviour *in vivo* is more than likely, but remains to be elucidated.

Other studies on glucose metabolism aimed at the characterization of end-products. Sutton and Starr (1959, 1960) determined that *E. amylovora* dissimilation of glucose produced mainly ethanol and carbon dioxide, with small amounts of lactic acid and acetic acid, formic acid, succinic acid, acetoin and 2,3-butanediol. It is, then, possible that *E. amylovora* possesses enzymes for dissimilation of glucose via the Embden–Meyerhof pathway. This is in contrast to other *Enterobacteriaceae*, which usually produce a large amount of formic acid. But the meaning of this for taxonomic comparisons is doubtful, since erwinias in general do not show a characteristic pattern of fermentation end-products (White and Starr, 1971).

Under aerobic conditions, metabolism of glucose by *E. amylovora* produced a high yield of 2-ketogluconic acid (Suzuki and Uchida, 1965a). This property was shared by several other *Erwinia* species, but not by *E. carotovora*. The most striking difference between *E. amylovora* and *E. carotovora* in this respect was that the latter accumulated ketoglutaric acid in the medium, without any accumulation of 2-ketogluconic acid (Suzuki and Uchida, 1965b).

Nitrogen metabolism

Mineral nitrogen

E. amylovora and some other *Erwinia* species, such as *E. ananas*, *E. mallotivora*, *E. nigrifluens*, *E. psidii*, *E. rhapontici*, *E. rubrifaciens*, *E. salicis*, *E. stewartii* and *E. tracheiphila*, do not reduce nitrate to nitrite, which is the general rule in *Enterobacteriaceae*. For this reason, this negative property received some attention. It has been suggested that this result could be an artefact, because

'*Erwinia amylovora* grows poorly in peptone nitrate medium' (Starr and Chatterjee, 1972). No experimental data, whatever the media or the techniques, showed reduction or respiration of nitrates by *E. amylovora*. On the contrary, this feature is selected among all minimal sets of determination of the bacterium in the laboratory (Billing *et al.*, 1961; Lelliott, 1967; Paulin and Samson, 1973). Among *Erwinia*, all species formerly placed in the '*carotovora*' group (Cowan, 1974) do reduce nitrate to nitrite, as do typical *Enterobacteriaceae*.

Organic nitrogen

The range of amino acids utilized as sole nitrogen source is indicated in several taxonomic studies (Slade and Tiffin, 1984; Verdonck *et al.*, 1987; Holt *et al.*, 1994). Results may vary widely according to techniques. Few studies attempted to comprehend the nutrition of the bacteria in plants. In this respect, Lewis and Tolbert (1964) showed that aspartate was both a nitrogen source for *E. amylovora* and a major component of amino acids (58%) in apple shoots.

Aminopeptidase profiles were established to compare strains. Differences between virulent and non-virulent strains were quantitative but not qualitative. No pattern typical for non-virulent strains could be proposed (McIntyre *et al.*, 1975).

Auxotrophy

The requirement for nicotinic acid was first pointed out by Starr and Mandel (1950). No other requirement for a growth factor was indicated, except for thiamine, which is required by a few wild-type strains (Bennett and Billing, 1978) and strains cured of the pEA29 plasmid. The need for nicotinic acid is not a common requirement among erwinias, and it was proposed as a biochemical test for *E. amylovora* characterization (Holt *et al.*, 1994).

Production of extracellular enzymes

Several enzymes, known in some cases to be involved in the plant-pathogen interaction, were specifically studied in *E. amylovora*, again in the hope of understanding the pathogenicity of this species.

β -Glucosidase

This enzyme is particularly interesting, because it is reported to result in the formation of glucose and hydroquinone from arbutin, a common compound in

apple. Formation of hydroquinone is significant, because it is toxic to bacteria (Hildebrand and Schroth, 1963). It was shown that *E. amylovora* exhibits weak β -glucosidase activity, which is strongly dependent upon the growth medium (Schroth and Hildebrand, 1965). Nevertheless, hydroquinone was demonstrated to be toxic to *E. amylovora* (Berg and Gibbins, 1983). It was assumed that it acts by inhibiting oxidation of succinate, D-lactate, DL-malonate and NADH (in the presence of oxygen) by cytoplasmic membrane and therefore inhibiting the growth of the bacteria. It could act on transport of electrons by ubiquinone. A ubiquinone, which could be the site of action of hydroquinone, was isolated from the cytoplasmic membrane of *E. amylovora* (Berg and Gibbins, 1983). In the presence of an exogenous β -glucoside, such as arbutin in apple tree tissues, this ubiquinone could act as a defence mechanism. Phloridzin was reported to play in pear tissues a role similar to that of arbutin (Gibbins, 1972). But this now seems less likely, since Kerppola *et al.* (1987) demonstrated that the pathogenicity of mutants of *E. amylovora* overproducing β -glucosidase was not affected by a 100-fold increase of β -glucosidase activity.

Hydrolytic enzymes

A number of pathogenic bacteria, especially among erwinias the '*carotovora*' group (Dye, 1969a) are 'macergens' (Billing, 1987), producing a massive quantity of cell wall polysaccharide-hydrolysing enzymes. In the case of 'necrogers' (Billing, 1987), the production of these enzymes, although in smaller amounts, is not exceptional. It is therefore a characteristic of *E. amylovora*, which is a necrogen, to produce no detectable amount of such enzymes, as has been shown by Seemuller and Beer (1976): no pectolytic, cellulolytic or xylolytic activity was detected during the development of the disease. Conversely, two neutral proteases were produced by the bacteria: one was isolated from the ooze of diseased plants and the other from infected plant tissue and culture medium (Seemuller and Beer, 1977). Their optimal pH of activity was 7.5 and 6.5, respectively. Their role in the infection process has not been established.

Secondary metabolites

In the search for toxic molecules produced by *E. amylovora* that could be responsible for pathogenicity, two original molecules have been isolated. One is 6-thioguanine (Feistner and Staub, 1986), which ultimately showed no toxic effect on pear cells in culture. In contrast, the other molecule, 2,5-dihydrophenylalanine (DHP) (Feistner, 1988), was considered to be a necrotoxin, although produced by both virulent and non-virulent forms of the pathogen. It was believed either to directly kill plant cells or to block induction of the hypersensitive reaction (HR) in infected plants. Nevertheless, although these are still possibilities, the lack of consistency of DHP production by diverse

strains of *E. amylovora* (Schwartz *et al.*, 1991) suggests that the role of DHP in virulence is not of key importance.

Motility

Like most phytopathogenic bacteria, and especially the erwinias, with the exception of *E. stewartii*, *E. amylovora* is motile in culture by media by means of two to seven peritrichous flagella per cell. This motility has been studied in detail by Raymundo and Ries (1980a, 1981). Synthesis of flagella is temperature-dependent (optimum 18–25°C); for maximal motility, pH 6.9 and the presence of a chelating agent, such as ethylenediaminetetra-acetic acid (EDTA) are required. Motility can be expressed in anaerobic conditions if a suitable carbon source is provided, but no motile bacterial cells are observed in the intercellular spaces of infected plant tissues. On the plant surface, the motility of *E. amylovora* is probably easily expressed: motile cells were seen under the microscope, within 10–30 s after their release from the stigma surface, by Thomson (1986). Interestingly, the motility in *E. amylovora* has been shown to be associated with a specific chemotaxis, which is temperature- and pH-dependent (optima: 20°C, pH 6.8). An original feature for the bacteria was that this chemotaxis was positive for one amino acid (aspartate) and some organic acids (fumarate, malate, maleate, malonate, oxaloacetate, succinate), but no chemotaxis was observed with any of the sugars tested (Raymundo and Ries, 1980b). In addition it was underlined that taxis for dicarboxylic acid was unique among bacterial plant pathogens, and that such acids were present in the nectar of apple flowers. Negative chemotaxis was shown for benzoate and salicylate and inducible negative chemotaxis for L-leucine, L-isoleucine and L-phenylalanine. Chemotaxis was believed to be due to a single bacterial receptor. A weak association between this property and pathogenicity was shown when *E. amylovora* was sprayed on apple blossoms, but not when inoculated with a needle into shoot seedlings (Bayot and Ries, 1986): more infections were obtained on apple blossoms sprayed with motile bacteria, while no difference was noted between shoot-inoculated seedlings, whatever the motility of the strain.

Plasmids

Plasmids are known to be present in a number of strains of phytopathogenic (and other) bacteria. In the case of *E. amylovora*, the same plasmid (pEA29) seems to be present in all investigated strains of the pathogen (Falkenstein *et al.*, 1989; Laurent *et al.*, 1989). This 29 kb plasmid seems to play a quantitative role in pathogenicity (Laurent *et al.*, 1989). But no precise functions were associated with its presence in the bacterial cell, except for a role in thiamine requirement (Laurent *et al.*, 1989). The presence of this plasmid in all strains of *E. amylovora* allowed primers specific to a DNA fragment of pEA29 to be proposed as a tool

for detection of *E. amylovora* by PCR (Bereswill *et al.*, 1992). A certain level of variation in length of the PCR product was found (Lecomte *et al.*, 1997), possibly linked with the geographical origin of the strain. Other variations were found in relation to strain origin (Momol *et al.*, 1997; Momol and Aldwinckle, Chapter 4) and possibly to the infected plant (*Rubus* strains). Up to now, no wild *E. amylovora* strain has been found lacking the pEA29 plasmid, but a few exceptions might exist. Two non-virulent strains of *E. amylovora* lacking pEA29 were described by J.L. Vanneste (unpublished results, 1993).

Sensitivity to antibiotics

Sensitivity or resistance to a determined concentration of antibiotics, in artificial medium, may be considered a characteristic of a bacterial species. The most comprehensive study of sensitivity or resistance of *E. amylovora* to antibiotics has been published by Vantomme *et al.* (1986): among 122 strains of *E. amylovora* tested, most were susceptible to: ampicillin (10 µg), cephaloridine (25 µg), chloramphenicol (30 µg), kanamycin (30 µg), nalidixic acid (30 µg), nitrofurantoin (200 µg) and streptomycin (10 µg), and resistant to: bacitracin (10 U), calistin sulphate (10 µg), erythromycin (10 µg), fusidic acid (10 µg), lincomycin (2 µg), methicillin (10 µg), penicillin G (10 U) and polymixin B (300 U), while variable responses according to strains were obtained for: gentamicin (10 µg), neomycin (30 µg), novobiocin (30 µg) and sulphafurazole (10 µg). Resistance to streptomycin is now common in natural bacterial populations exposed to multiple sprays of this antibiotic during normal control strategies in certain countries (Jones and Schnabel, Chapter 12).

Serology

Serology has been used for a long time in the study of *E. amylovora* for diagnostics, improved description of the species, assessment of its homogeneity and differentiation from other supposed closely related species. Internationally accepted specific reagents were needed for diagnosis and detection of *E. amylovora*, because of its limited geographical distribution and because it is a quarantined pathogen in several countries. Specific antisera and serological techniques were good candidates for this purpose. Data on serological characteristics of *E. amylovora* were first obtained with polyclonal antibodies but, soon after the discovery of monoclonal antibodies (MCAs), more studies were published.

Serological properties

The most comprehensive study of serological properties of the genus *Erwinia* has been presented by Slade and Tiffin (1984). Former works of some importance

(i.e. dealing with several strains of each species) were those of Elrod (1941) and Lazar (1972b). The usefulness of serological studies for bacterial plant pathogens, in the case of fire blight, was pointed out as early as 1933 (Rosen and Bleecker, 1933).

It is rather difficult to get a general view from these studies because techniques differ, from the very preparation of the antigen (living bacteria, heated cells, etc.) to the visualization of the specific antigen-antibody reaction (tube agglutination, agglutinin absorption, immunodiffusion, etc.). Nevertheless, the general tendency is to describe *E. amylovora* as a serologically homogeneous species (Elrod, 1941; Lazar, 1972b). A tentative establishment of serotypes within the species, based on surface antigens (Samson, 1972), has not been studied further. Among erwinias, the work of Lazar (1972b), using both cross-agglutination and double gel diffusion, tended to conclude that there was a close serological relationship between the species included in the genus *Erwinia* (nine species). Unfortunately, in the double gel diffusion part of this work, none of the five *E. amylovora* strains used in cross-agglutination were included. It is noteworthy that, in the agglutination tests, all the five isolates of *E. amylovora* agglutinated in the same antisera, thus confirming the serological uniformity in *E. amylovora*. Nevertheless, these *E. amylovora* reacted to a certain extent with antisera produced against other erwinias, sometimes giving obvious positive reactions (e.g. with antiserum prepared against *E. aroideae* or *E. chrysanthemi*). It is possible that the techniques used were not really suitable for establishing relations between erwinias: it is uncertain what a cross-reaction between two organisms implies.

The use of MCAs might produce some new information with respect to the possible heterogeneity of *E. amylovora* as a group and the relations between erwinias. It is now possible, with MCA, to identify a unique antigenic determinant on the cell surface, thus theoretically allowing a very precise study of cell surface antigens. In addition, taxon-specific MCAs have been isolated for xanthomonads, showing the potential of this new tool (Alvarez and Benedict, 1990). This type of study has not yet been undertaken for *E. amylovora*. Rat and mice MCAs have been produced (Hutschemackers *et al.*, 1987b; Lin *et al.*, 1987; McLaughlin *et al.*, 1989; Gugerli and Gouk, 1994; Gorris *et al.*, 1996b) and sometimes shown to be specific for *E. amylovora* when compared with other plant-pathogenic and saprophytic bacterial species. Usually, a mixture of several MCAs was necessary to allow recognition of all the representatives of *E. amylovora* (Gugerli and Gouk, 1994; Gorris *et al.*, 1996b). This indicates that some epitopes are not found on every strain, and shows the possibility of description of further antigenic variability.

Serological structure

The diverse antigens that compose the antigenic capacity of *E. amylovora* are relatively complex (Slade and Tiffin, 1984). Several antigens were distinguished

and could be prepared independently from pure culture: LPS (see above for composition and structure), which can be either rough or smooth (with or without a side-chain); a neutral heat-stable antigen, named GAI, which was thought to be a polysaccharide distinct from LPS, common to all representatives of the '*amylovora*' group; antigen TV, present only in virulent *E. amylovora*, was likely to be part of the EPS; another complex antigen, GAJ, was detected in the extracellular slime from bacterial culture. An attempt to correlate serological feature and virulence failed to show more than the formerly established link between capsular material and pathogenicity. Using immunodiffusion after chemical treatment of antigens from *E. amylovora*, cross-reactions were found between capsular antigens of *E. amylovora* and *E. herbicola* (Slade and Tiffin, 1978). Former studies had shown that induced non-pigmented variants of *E. herbicola* might have some serological relationship with *E. amylovora* (Gibbins, 1974), but these common antigens were not of capsular origin.

In spite of all theoretical considerations on antigens of *E. amylovora*, the search for specific antigens remains more or less empirical. As far as preparation of antiserum is concerned, very few works deal with a comparison of procedures according to the type of serum (agglutinant or precipitant) that is needed. Such a study was published by Lazar (1972a) for diverse *Erwinia*. It indicated the best procedure to be used in each case; in addition, it showed that *Erwinia* was highly toxic for rabbits (except *E. atroseptica*). The antigens used to prepare *E. amylovora* antisera are very diverse, as are the immunization protocols: living cells, heat-killed cells, formalin-treated cells, the supernatant of killed cells or much more complex extracts from the bacteria were tested. Laroche and Verhoyen (1986) demonstrated that the supernatant of cells treated with phenol contained a specific antigen, as seen by immunodiffusion. Analysis revealed that it was in fact a lipopolysaccharide whose antigenic structure involved a polysaccharidic function.

Serological techniques for diagnosis and detection

Agglutination

Slide agglutination, following a precise procedure (Lelliott, 1967), is an easy test that usually provides good results. It relies on the high homogeneity of thermostable antigens within *E. amylovora*. Nevertheless, cross-reactions with diverse bacteria are sometimes observed (*Pseudomonas syringae*, *E. herbicola*) (Billing *et al.*, 1960; Israily *et al.*, 1966) and additional tests are recommended for good identification (Paulin and Samson, 1973; Miller, 1979; Van Vaerenberg *et al.*, 1987).

Immunodiffusion

Immunodiffusion techniques are not normally advised for standard diagnostic applications. Nevertheless, in a suitably equipped laboratory, they were recommended for this purpose, with reliable results (Laroche and Verhoyen, 1982).

Immunofluorescence

Immunofluorescence (IF) is a standard technique, used for detection of bacteria in complex media, in which specifically marked bacterial cells are directly observed through the microscope. For *E. amylovora*, it has been used with success for monitoring the bacterial population on the plant surface (Thomson and Schroth, 1976; Miller, 1983), for diagnosis (Roberts, 1980; Calzolari *et al.*, 1982) and for localizing bacteria in plant tissues, in connection with histological studies (Hockenhull, 1978).

As with all detection methods, problems with specificity and sensitivity can occur. As far as specificity is concerned, it obviously depends on the quality of the antiserum used. Cross-reactions have been assessed with a number of bacteria likely to be found on the plant surface or associated with lesions: *E. herbicola*, *Erwinia uredovora*, *E. rhapontici*, *Pseudomonas fluorescens*, *Citrobacter* spp. (Calzolari *et al.*, 1982). These cross-reactions could be reduced by the use of diluted antisera, but this involves the risk of false-negative responses with some strains of *E. amylovora*. The same was previously experienced by Roberts (1980), who proposed the simultaneous use of different antisera, prepared from different antigens of *E. amylovora*, to tackle the difficulty.

Utilization of a given mixture of specific monoclonal antisera could be helpful in this case. Ten specific MCAs were tested successfully in IF by Lin *et al.* (1987). Hutschemackers *et al.* (1987b) used three specific MCAs, which were shown to be strictly specific for *E. amylovora* (93 strains). In this case, no reactions were observed with 88 strains of other bacterial species. In addition, the particular advantage of MCA, especially for a test which could be marketed, is its theoretical constant quality and availability (Hutschemackers *et al.*, 1987b). Direct examination of bacteria by IF from plant tissues (Laroche and Verhoyen, 1983) was successful, with a pretreatment of samples to reduce the naturally occurring plant fluorescence. The observation could also be performed after printing the leaves on a collodion film, on which the serological reaction and microscopic examination were performed. However, whatever the technique, the minimal number of bacteria must be as high as 10^6 – 10^7 cells ml⁻¹, to allow a positive detection in IF (Laroche and Verhoyen, 1983).

ELISA

Numerous ELISA techniques have been used, or rather tested, extensively for detection of *E. amylovora*. They are usually preferred to IF, for practical reasons; ELISA can be made automatic, the assessment of results is less subjective

(optical density) and serial analyses in the laboratory are far less time-consuming than they are with IF.

Adaptation of ELISA to *E. amylovora* detection was first proposed by Laroche and Verhoyen (1984). An improvement of the method, whose specificity was not high enough, was further indicated by the same team (Laroche *et al.*, 1987). Supernatants of *E. amylovora* cultures were used as a source of antigens and tested with a large number of strains of *E. amylovora* (91). A specific reaction was obtained from *E. amylovora* metabolites, even when they were mixed with other bacteria.

In order to reduce the risks of non-specific response (higher with ELISA than with IF, because there is no direct observation of marked cells), ELISA is now used in association with MCAs.

The use of a mixture of three specific MCAs, each bound to a unique epitope, allowed McLaughlin *et al.* (1989) to obtain a reagent both specific in ELISA and sensitive enough to detect about 10^5 – 10^6 cells ml⁻¹. The threshold for detection using an MCA depends on the type of ELISA technique used; for pure culture, it is considered to vary from 10^6 cells ml⁻¹ to 10 cells ml⁻¹ (Hutschemackers *et al.*, 1987a). In contrast to previous results, Gugerli and Gouk (1994) found a high proportion of MCAs that cross-reacted with *E. herbicola*, suggesting common epitopes. Nevertheless, *E. amylovora* was readily distinguished (in indirect ELISA) from other genera of bacterial pathogens. As far as *E. amylovora* was concerned, different reactivity patterns were obtained, suggesting strain-specific epitopes. Gorris *et al.* (1996b) prepared MCAs using two types of antigens: EPS from one *E. amylovora* strain and cells of a non-capsulated derivative. Eight MCAs were obtained from the two antigens: three from EPS and five from the whole cell. Most were specific for *E. amylovora*. They represented at least five different native epitopes, according to the pattern obtained with 48 *E. amylovora* strains. Two selected MCAs reacted in the different techniques used: indirect ELISA, ELISA double antibody sandwich indirect (DASI), with native or boiled antigens. These reagents and techniques were further proposed in association with enrichment on suitable media for a specific and sensitive detection of *E. amylovora* (Gorris *et al.*, 1996a), as formerly suggested by Hutschemackers *et al.* (1987a). The sensitivity, which was assessed at 10^5 cells ml⁻¹ without enrichment, could decrease to 10 cells ml⁻¹ following enrichment.

Other techniques

Techniques for visualization *in situ* of bacterial cells, such as immunogold staining (IGS) or immunogold silver staining (IGSS), have been used with success, and gave rise to a permanent marking of bacterial cells (Van Laere *et al.*, 1985). This was an advantage compared with IF, whose markings tend to fade away with time and observations. It could therefore be recommended for precise histological studies. In addition, IGSS gave a high contrast in normal microscopy, which is another advantage compared with UV microscopy, used for IF.

Two other techniques (immunoelectrotransfer, immunoprinting) were tested successfully for *E. amylovora* by Gorris *et al.* (1996a). The very simple and convenient technique of immunoprinting, which is based on the same capture procedure as print-capture PCR (Olmos *et al.*, 1996) – pressure of freshly cut plant tissues, such as a leaf petiole, on to suitable blotting paper – allows easy sampling in the field and delayed serological reaction in the laboratory. It may also be very promising as a practical detection tool for *E. amylovora*.

Sensitivity to bacteriophages

Bacteriophages, or phages, are viruses that specifically infect their bacterial host. Therefore, susceptibility of several bacteria to a virus often indicates at least common receptors on the surface of the bacterial envelope. This may also indicate a certain level of similarity between these bacteria. On the contrary, differences in susceptibility to the same phage between bacteria otherwise similar (i.e. from the same species) would indicate tiny but sometimes significant differences between them. Such a difference was found by Billing (1960) between non-virulent and virulent *E. amylovora*. She found one phage that gave no clear lysis with cultures, showing both atypical colonies and low virulence. The susceptibility to phages was dependent on the presence of the capsule around the cells. Later, it was shown that LPS, which is not a key factor in virulence, could be responsible for phage specificity (Billing, 1985).

Comprehensive studies on the phage susceptibility of *E. amylovora* were undertaken in the search for a diagnostic tool. An analysis, with seven phages, of 616 strains of Gram-negative bacteria isolated from orchards diseased with fire blight was produced by Hendry *et al.* (1967). It showed that all the 194 *E. amylovora* strains, 94 yellow and 26 white isolates, were lysed by at least one phage. *E. amylovora* strains were distributed into seven different patterns of phage sensitivity, but no phage appeared to be specific to only *E. amylovora*. Later, Ritchie and Klos (1979) described 11 phages isolated from the aerial part of apple trees. All were showing a host range limited to *E. amylovora* and few strains of *E. herbicola*. Most phages showed plaques surrounded by an expanding halo, due to the hydrolysis of bacterial EPS by a phage hydrolase. The sensitivity of *E. amylovora* and *E. herbicola* to the same phages was similarly noted. The use of the latter bacterial species on plants as a reservoir of phages for biological control had formerly been proposed (Chatterjee and Gibbins, 1971). The possible role of phages in fire blight epidemiology was considered by Erskine (1973): he suggested that the spontaneous release of phages from the lysogenic form of a 'yellow *amylovora*-like saprophyte' was able to modulate the severity of fire blight and the occurrence of the disease, in controlling the population of *E. amylovora* susceptible to these phages.

Utilization of phages has been suggested (Billing *et al.*, 1960; Hendry *et al.*, 1967; Paulin and Samson, 1973; Vanneste and Paulin, 1990) as a complementary tool for diagnosis. But the fact that a single mutation may change a

susceptible cell to a resistant one may be a cause for false-negative results. In addition, no phages specific to all the *E. amylovora* strains tested were found. We note here a tentative determination of *E. amylovora* from bacterial populations in contaminated apple buds (Baldwin and Goodman, 1963), which relied mainly on phage sensitivity, and which described 'white virulent', and 'yellowish non-virulent' isolates. This finding reinforced the already existing assumption that, in a diseased tree, two forms of the pathogen could cohabit, with possible conversion between these two forms (for a review, see Gibbins, 1972). This relied on a number of studies comparing *E. amylovora* and *E. herbicola* (Chatterjee and Gibbins, 1971) in physiology and serology (some of which have been presented in this chapter). We now have enough information to recognize that this assumption was probably not accurate: it is likely that the white virulent strains were *E. amylovora* and that the yellowish non-virulent strains were *E. herbicola* – two distinct species but both frequently sensitive to the same phages.

A most important contribution of phages to the study of *E. amylovora* (and other pathogenic bacteria) is the discovery and use of mutator phages. The mutagenesis of strain 1430 of *E. amylovora* by a modified phage Mu (Vanneste *et al.*, 1990) was at the origin of part of the work on the genetic analysis of pathogenicity (Barney *et al.*, 1990).

Summary

E. amylovora can be considered both as a well-known bacterial species and as a poorly known bacterial plant pathogen.

The first comment can be made because of the large amount of data collected on its anatomical, physiological and serological characters. The description is precise enough to indicate a well-defined group of bacteria, which clearly constitutes a species. This is confirmed by molecular studies. Nevertheless, the place of this species in bacterial taxonomy is not so firmly settled, and it is uncertain if this *amylovora* species will remain in the genus *Erwinia*. Genomic analysis of these bacteria could provide useful new data in this respect. On the other hand, recent molecular results, as well as pathogenicity studies, would tend to weaken the strong image of homogeneity provided by other studies (Momol and Aldwinckle, Chapter 4): are we moving towards the description of pathovars within the species *E. amylovora*?

The second assessment relies on the lack of clear-cut differences found in physiology, serology and other characters between non-virulent and virulent forms of the pathogen, with the very noticeable exception of the bacterial EPS in certain cases. Genetic and molecular studies now in progress are rapidly providing an enormous amount of invaluable information in this field, as indicated in the following chapters.

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Exopolysaccharides of *Erwinia amylovora*: Structure, Biosynthesis, Regulation, Role in Pathogenicity of Amylovoran and Levan

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Exopolysaccharides and fire blight

Ooze produced by *Erwinia amylovora* on plant surfaces consists of exopolysaccharide (EPS) and bacteria, which are intimately connected, and allows fire blight to establish and spread (Bennett and Billings, 1978; Ayers *et al.*, 1979). In addition to necrotic symptoms, droplets of ooze are often seen as a striking sign of fire blight. When *E. amylovora* enters plant tissue, the bacteria have a strong tendency to invade the xylem (Bogs *et al.*, 1998; Vanneste and Eden-Green, Chapter 5). Bacteria moving through the plant vessels can result in bacterial aggregations, mediated presumably by local accumulation of EPS and disruption of the water flow (Sjulin and Beer, 1977). This causes leakage of the vessels and extrusion of bacteria into the parenchyma and finally forces ooze out to the plant surface. In addition to the complex EPS amylovoran, the bacterial exudate may contain the homopolymer levan. *E. amylovora* strains without the capacity to synthesize amylovoran are non-pathogenic (Steinberger and Beer, 1988; Bernhard *et al.*, 1993); they do not multiply in plants (Bellemann and Geider, 1992) and are unable to move in the vessels (Bogs *et al.*, 1998). Levan-deficient strains are only affected in virulence (Geier and Geider, 1993).

A good source of amylovoran from plant tissue is inoculated pear slices. When bacteria are removed from the ooze, the EPS is identical in structure and similar in molecular weight to material from bacteria grown on agar plates (Langlotz *et al.*, 1999).

The amount of EPS synthesized on plates can vary for different *E. amylovora* strains. Environmental factors, such as temperature, the pH of the medium, its salt concentration and the carbon source, also affect amylovoran synthesis. When grown on minimal media with sorbitol, bacteria produce more EPS than

when grown on nutrient broth-based media. Even in the latter case, sorbitol increases EPS production. Galactose has an even stronger effect than sorbitol (Bellemann *et al.*, 1994). The presence of copper ions in the medium also increases the level of amylovoran synthesis and induces the formation of yellow mucoid colonies, a characteristic that can be used for identification of *E. amylovora* (Bereswill *et al.*, 1998).

Functions and structures of polysaccharides of *E. amylovora* and other bacteria

Bacterial polysaccharides can be intimately linked to the cells, like lipopolysaccharide (LPS), or form loose capsules, like EPS. LPS has a strong influence on the serological characteristics of a species and is responsible for classification in serotypes (Roberts, 1995; see also Paulin, Chapter 6). In contrast, EPS is barely immunogenic. This property allows pathogens to elude host recognition and escape host defences. Capsulated cells, in contrast to EPS mutants, were not affected by agglutinins from apple seeds (Romeiro *et al.*, 1981), but EPS-deficient and wild-type *E. amylovora* strains were equally able to induce electrolyte leakage in host plant tissue (Brisset and Paulin, 1992). EPS capsules also prevent cells from losing water, which can be important under dry environmental conditions (C. Langlotz and K. Geider, unpublished). Charged EPS can bind ions, which might be beneficial in increasing ion concentration in the neighbouring cells. Although the binding by charged EPS may include ionic nutrients, no good evidence has been presented that the EPS may be an external energy storage system, in contrast to plants, which use starch and other polymers to provide the organisms with energy when needed.

Levan is an EPS with a single sugar, a homopolymer of fructose residues. Many plant-associated bacteria secrete an enzyme that cleaves sucrose in the environment, thereby releasing the glucose and polymerizing the fructose residues into β -2,6-fructan (levan) (Fig. 7.1). Since plants produce large amounts of sucrose during photosynthesis and use it as a transport sugar, phytopathogenic bacteria readily find a substrate for levan synthesis. Levan may be easily synthesized following secretion of the polymerizing enzyme levansucrase, but seems to lack a lasting effect in shielding pathogens from unfavourable environmental conditions.

Plant-associated bacteria produce complex EPS, whose composition and sugar linkages are characteristic of a bacterial species. The polymerization of a repeating unit results in high-molecular-weight EPS. The structure of the repeating unit from amylovoran of *E. amylovora* is outlined in Fig. 7.2; it consists of a backbone of three galactose residues and a side-chain of glucuronic acid and another galactose residue, which is substituted by acetyl groups and pyruvate. Stewartan, the EPS produced by *Erwinia stewartii*, the EPS from *Erwinia chrysanthemi*, xanthan from *Xanthomonas campestris*, the alginate from *Pseudomonas aeruginosa*, the succinoglycan (EPS I) from *Rhizobium meliloti* and

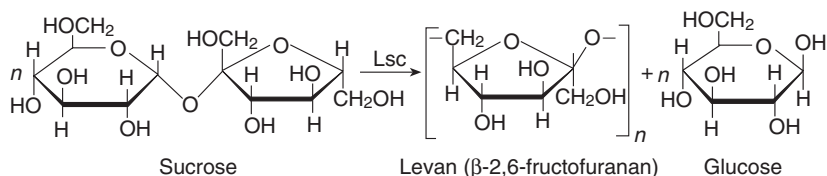


Fig. 7.1. Synthesis of levan by levansucrase (Lsc) from sucrose in the bacterial environment. The constitutively secreted enzyme attaches to the growing chain of the polymer. Levan could protect the cells and the released glucose could be used as a carbon source.

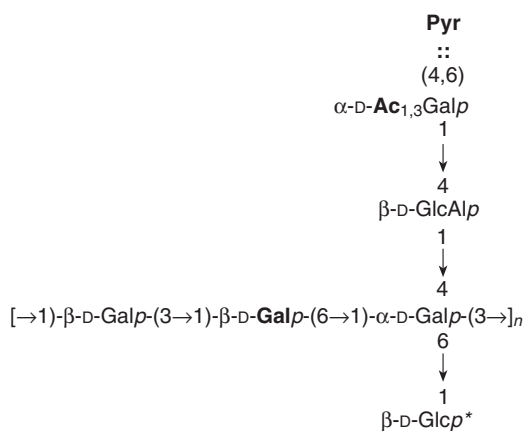


Fig. 7.2. Structure of the repeating unit of amylovoran. The structure is a mirror of the original structure published by Nimtz *et al.* (1996a). Changes in comparison with the repeating unit of stewartan (see Fig. 7.3) are indicated in bold face, where the central galactose of amylovoran is substituted by glucose, the pyruval residue by galactose ($\beta 1 \rightarrow 6$) and the acetyl residues are lacking.

* About 10% of repeating units carry a glucose as a second side-chain (in the case of stewartan, 90%). Gal, galactose; GlcA, glucuronic acid; Pyr, pyruval residue with keto linkage; α , β , sugar linkage at C1; D, sugar configuration; p: pyranoside; n, degree of polymerization (at least 1000).

the colanic acid from *Escherichia coli* also have specific repeating units and sugar linkages (Fig. 7.3). A comparison of the repeating units shows a certain level of diversity. Amylovoran and stewartan are highly similar, differing only in one sugar in the backbone and the terminus of the side-chain.

Alginate of *P. aeruginosa*

copolymer of mannuronic acid
and glucuronic acid

EPS of an *E. chrysanthemi* strain

-glucose-mannose-rhamnose
rhamnose-rhamnose-glucose

Colanic acid of *E. coli*

-fucose-glucose-fucose-
|
galactose
|
glucuronic acid
|
galactose-pyruvate

Xanthan of *X. campestris*

-glucose-glucose-
|
mannose
|
glucuronic acid
|
mannose-pyruvate

Succinoglycan of *R. meliloti*

-glucose-glucose-glucose-galactose-
|
glucose
|
glucose
|
glucose
|
glucose-pyruvate

Stewartan of *E. stewartii*

glucose
|
galactose
|
glucuronic acid
|
-galactose-glucose-galactose-
|
glucose

Fig. 7.3. An outline of basic structures for bacterial exopolysaccharides (EPSs). Details about their detailed structures, biosynthesis and genetics were discussed in the reports by Govan and Deretic (1996) for alginate of *Pseudomonas aeruginosa*; by Garegg *et al.* (1971) and Keenleyside *et al.* (1992) for colanic acid of *Escherichia coli*; by Gray *et al.* (1993) for EPS of *Erwinia chrysanthemi* strain SR260; by Sutherland (1988) for xanthan of *Xanthomonas campestris*; by Reuber and Walker (1993) for succinoglycan of *Rhizobium meliloti*; and by Nimtz *et al.* (1996b) and Yang, B.Y. *et al.* (1996) for stewartan of *Erwinia stewartii*.

Properties of amylovoran and levan

The sugar linkages and the molecular weight of amylovoran and levan were determined by several approaches. The size of the molecules was assayed by size exclusion chromatography (SEC), multi-angle light scattering (MALS) and for amylovoran also by analytical ultracentrifugation (Jumel *et al.*, 1997). SEC relies on the elution profile of the molecules from large-pore gel permeation columns. Low-angle light scattering depends on the ability of large molecules to reflect light. Analytical ultracentrifugation has been used for many years for sedimentation of large macromolecules. With these three methods, the molecular weight of amylovoran from strain Ea1/79, which was freeze-dried for storage, was determined to be approximately 1×10^6 Da. This corresponds to about 1000 repeating units per EPS molecule. The size is dependent on growth conditions, but not much on individual strains (Langlotz *et al.*, 1999). Growth of *E. amylovora* on agar plates at 18°C seems to increase the molecular weight of

amylovoran, compared with growth at 28°C, probably due to different rates of chain termination for these conditions. Mechanical stress in culture suspension releases the EPS attached to the cells as a capsule, which could affect chain length as found for processing such as freeze-drying. The molecular weight of levan for strain Ea1/79 was determined to be $c. 5 \times 10^6$ Da (M. Schollmeyer, A. Huber and K. Geider, unpublished).

The precise structure of the repeating unit of amylovoran – the order and the linkages of sugar residues – was determined by several methods, such as sugar composition, methylation analysis and nuclear magnetic resonance (NMR) (Nimtz *et al.*, 1996a). Characteristic signals from proton-coupling and 2D NMR also allowed the assignment of the α and β linkages. Additional hints for α -linked sugars were obtained by cleavage in liquid hydrofluoric acid. NMR analysis required the isolation of monomers and dimers of the repeating units. This was achieved with viral EPS depolymerase and separation by gel permeation chromatography.

Levan was also identified by NMR (Gross *et al.*, 1992). The signals for β -2,6-fructose linkages were different from the NMR spectra of β -1,2-inulin.

Genetics of amylovoran biosynthesis

Because of its complex sugar linkages (Fig. 7.2), biosynthesis of amylovoran requires a large number of genes. Most of the structural genes are located in an approximately 17 kb region of the chromosome called *ams* (Bugert and Geider, 1995). The 12 open reading frames (ORFs) that comprise the *ams* region are surrounded by nucleotide sequences for genes unconnected to EPS synthesis, such as *udk* (uridine kinase) and *dcd* (dCTP deaminase) to the left and *rfbB* (TDP-glucose oxidoreductase, TDP-glucose-4,6-dehydratase) to the right. Two genes, *amsM* (analogous to *galF*) and *galE* (UDP-galactose epimerase), which are located on the right adjacent to the *ams* cluster, are involved in the formation of precursors (Fig. 7.4). The involvement of the individual ORFs of the *ams* region in EPS synthesis was shown by analysis of mutants obtained by insertion of a transposon or of a resistance cassette (Bernhard *et al.*, 1993; Bugert and Geider, 1995). For *amsG*, *amsH* and *amsI*, another series of mutations confirmed the requirement of these genes for amylovoran production (Menggad and Laurent, 1998). The products of all 12 genes are thus involved in individual steps of amylovoran synthesis and the two adjacent genes, *amsM* and *galE*, in precursor formation.

Functions of genes and the localization of the corresponding proteins can be estimated by computer search for homology in data libraries and consensus structures, such as helix–turn–helix (HTH) motifs or ATP-binding domains (Table 7.1). Association with cell membranes is likely for AmsH, A and L, and sugar transferase activities are likely for AmsG, B, C, D, E, J and K. AmsI has the function of an acid phosphatase (Bugert and Geider, 1997). AmsA acts as a tyrosine kinase (Ilan *et al.*, 1999), which could be involved in protein phosphorylation during transport of the repeating units, and subsequent

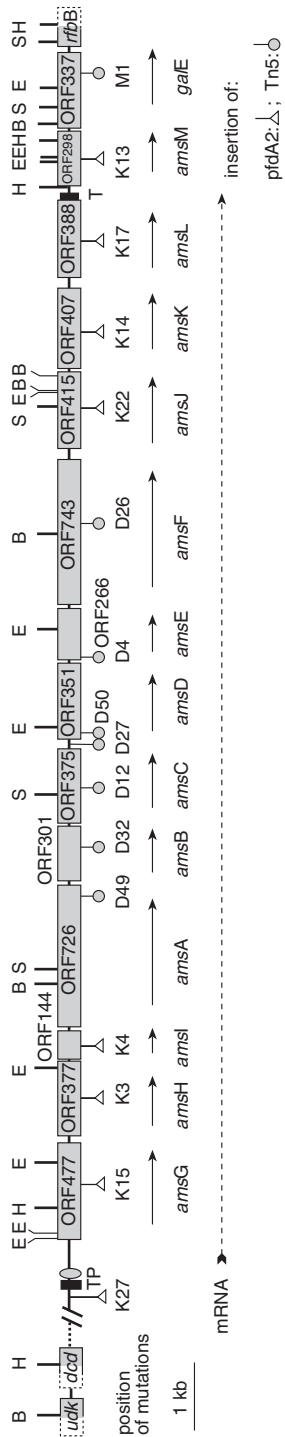


Fig. 7.4. Map of the *ams* region encoding amy/lovoran synthesis. The 16 kb transcript of the *ams* operon is shown at the bottom. P, promoter; T, terminator; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sal*I. (Mainly based on data from Bugert and Geider, 1995.)

Table 7.1. Properties of the proteins encoded in the *ams* region (derived from Metzger *et al.*, 1994; Bugert and Geider, 1995).

	Gene													
	amsG	amsH	amsI	amsA	amsB	amsC	amsD	amsE	amsF	amsJ	amsK	amsL	amsM	galE
Size of product ^a	477	377	144	726	301	375	351	266	743	415	407	388	298	337
Molecular mass (kDa)	55.1	41.4	15.7	80.4	34.7	42.5	39.8	30.8	82.2	46.4	45.1	43.7	32.7	36.7
Estimated pI (pH)	9.4	6.2	8.3	5.8	8.9	9.5	9.2	8.9	6.7	7.8	7.0	10.2	5.3	5.1
Homology to	RfbP	BexD	LMW-phosphatase	–	ExoO, U	–	RfaB	Lsg6	–	–	ORF0.0	–	GalF, U	Other GalE
Similarity (%)	78	45	39	–	45	–	33	53	–	–	68	48–81	38–64	–
Special sites in	4 tr. hel.	2 tr. hel. 1 lip. att.	–	4 tr. hel. 1 ATP-b.	–	4 tr. hel.	–	–	1 sss	1 tr. hel.	1 tr. hel.	8 tr. hel.	–	–
Probable localization	Mem-brane	Outer membrane	Cyto-plasm	Mem-brane	Cyto-plasm	Mem-brane	Cyto-plasm	Cyto-plasm	Peri-plasm	Mem-brane	Mem-brane	Mem-brane	Cyto-plasm	Cyto-plasm
Possible function	Gal transfer to lipid carrier	Transport of repeating unit in OM	Acid phosphatase ^b	ATPase of ABC-trans-porter ^c	Sugar trans-ferase	Sugar trans-ferase	Sugar trans-ferase	Sugar trans-ferase	Poly-merization	Sugar trans-ferase	Sugar trans-ferase	Pore formation	Subunit for GalU	UDP-gal syn-thesis

^a As amino acid.

^b Enzyme activity confirmed in phosphatase assay (Bugert and Geider, 1997).

^c Tyrosine kinase (Ilan *et al.*, 1999).

LMW, low molecular weight; tr. hel., transmembrane helices; lip. att., lipid attachment sites; ATP-b, ATP binding site; sss, secretory signal sequence; ABC, ATP binding cassette; OM, outer membrane.

^{14}C -UDP-galactose (Langlotz *et al.*, 1999). The *amsD* gene product catalysed the linkage of the next galactose residue to the galactose attached to the lipid carrier. The transferase activity encoded by *amsC* was required to couple the next galactose. Subsequent sugar attachment depends on the functions of *amsB*, K, E and J. The proteins required for processing, translocation and finally polymerization of the repeating units are presumably associated with *amsH*, A, F and L. The localization of AmsH and AmsF was shown by translational fusions with a *blaM* gene, not encoding a signal peptide, to be adjacent to or in the periplasm (H. Ullrich and K. Geider, unpublished). Since those fusions expressed β -lactamase activity, the corresponding *ams* gene products are most probably localized in the membrane. The final polymerization step is assumed to occur by the attachment of the linear repeating unit to an existing amylovoran chain. The mode of translocation may be similar to the growth of peptides catalysed in ribosomes (Bastin *et al.*, 1993). A functional association of a gene product with a sizing step has rarely been achieved in biochemical studies of EPS synthesis. In the case of colanic acid, a model for chain length determination was suggested, and the *cld* (*wzz*) gene was associated with this function (Franco *et al.*, 1996).

Since many bacteria apparently share principles and gene functions for polysaccharide synthesis, a general designation scheme was proposed for genes involved in LPS and EPS synthesis (Reeves *et al.*, 1996). Further analysis of the role of gene products in bacterial polysaccharide synthesis are required in order to describe functions of the genes involved in the synthesis of a particular EPS.

Closely related exopolysaccharides from *Erwinia pyrifoliae* and *E. stewartii*

In a recent survey in orchards near Chuncheon in South Korea, necrotic symptoms were discovered on the Asian pear tree (*Pyrus pyrifolia*). The causal agent was characterized as a Gram-negative bacterium with a close relationship to *E. amylovora* by microbiological, immunological and even some molecular criteria (Kim *et al.*, 1999; Rhim *et al.*, 1999). Its colony morphology on MM₂Cu agar (see Bereswill *et al.*, 1998) was mucoid, as for *E. amylovora*, although colonies had only a faintly yellow colour. No signals were obtained in PCR assays with the plasmid primers and the *ams* primers from *E. amylovora* (Rhim *et al.*, 1999). On immature pears, the pathogen isolated from Asian pears and named *E. pyrifoliae* produced ooze like *E. amylovora*. Assays on plants revealed a preference for symptom formation on pear seedlings, especially Asian pear (Rhim *et al.*, 1999).

Nucleotide sequence analysis of the 16S rRNA revealed more than 99% identity with the same gene from *E. amylovora*. The intergenic spacer region between the 16S and 23S rRNA genes of these two species showed a weaker homology, although *E. pyrifoliae* is closer to *E. amylovora* than to other erwinias (Table 7.2).

Table 7.2. Relationship of *Erwinia amylovora*, *Erwinia pyrifoliae*, *Erwinia stewartii*, *Escherichia coli* and other erwinias deduced from nucleotide sequences of the 16S rDNA and the 16S/23S rDNA spacer (data in part from Kim *et al.*, 1999; W.-S. Kim and K. Geider, unpublished).

Species	16S rDNA (% identical residues)	ITS (% identical residues)
<i>E. amylovora</i>	100	100
<i>E. pyrifoliae</i>	99	85
<i>E. stewartii</i> ^a	97	86
<i>E. ananas</i> ^a	97	
<i>E. herbicola</i> ^a	97	
<i>Escherichia coli</i>	97	74
<i>E. carotovora</i> subsp. <i>carotovora</i>	96	
<i>E. chrysanthemi</i>	96	
<i>E. salicis</i> ^b	94	

^a Now to genus *Pantoea* sp.

^b Now to genus *Brennenia*.

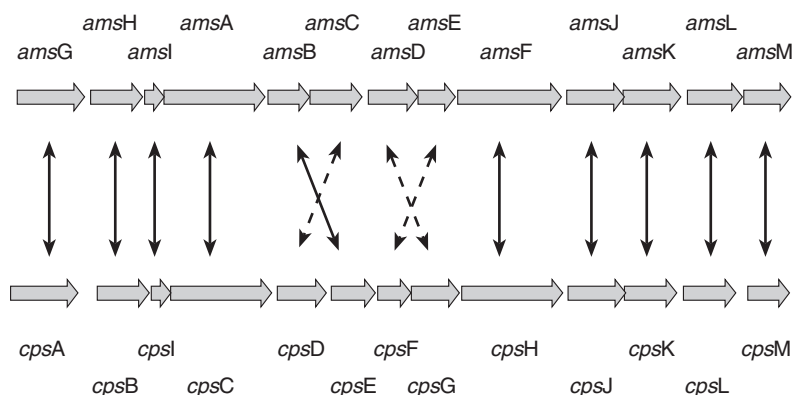
ITS, intergenic transcribed spacer between 16S and 23S rRNA.

EPS of *E. pyrifoliae* was isolated from mucoid colonies and its sugar composition and linkages were determined. Methylation analysis indicated that one glucuronic acid and four galactose residues build the repeating unit and, even more surprisingly, the sugars are linked to each other, as in amylovoran (M. Nimtz, W.S. Kim and K. Geider, unpublished). In a first study of the chromosomal region responsible for EPS synthesis in *E. pyrifoliae*, several oligonucleotides of the *ams* operon of *E. amylovora* were used for amplification of the corresponding regions of *E. pyrifoliae* (W.S. Kim and K. Geider, unpublished). Primers from the left part of the *ams* operon gave more positive bands than primers from the right part of the operon. DNA fragments with part of the *amsB* analogue and part of the *amsC* analogue were cloned and sequenced. Nucleotide residues were 95% and 88% identical, respectively, and the deduced amino acid sequences of the sequenced parts of AmsB and AmsC of *E. pyrifoliae* had 95% and 98% similarity, respectively, with AmsB and AmsC of *E. amylovora* (W.S. Kim and K. Geider, unpublished). It can be assumed that the gene products have the same catalytic functions in both species. When the *amsB* analogue gene of *E. pyrifoliae* was mutated, the strain had the same non-pathogenic phenotype as an *E. amylovora* *amsB* mutant. As sugar transferases are often quite divergent for bacteria, even when they catalyse a similar step in EPS synthesis (see Table 7.1), this homology is highly significant. This adds additional data about the relationship between *E. pyrifoliae* and *E. amylovora*.

E. stewartii is a vascular pathogen of maize. Based on its yellow pigment and on other properties, it has been recently reclassified into the genus *Pantoea* (Mergaert *et al.*, 1993). However, this species shares similarities with *E. amylovora* and will therefore be called here by its old name *E. stewartii*. A high

similarity is obvious from the organization, structure and sequence of the EPS-encoding region of the two species. Fourteen ORFs are arranged for *E. stewartii* in the order of *cpsA*, B, I, C, D, E, F, G, H, J, K, L and M followed by *galE* (Coplin *et al.*, 1996). The high similarity of most *cps* genes to the corresponding *ams* genes implies similar functions in EPS synthesis (Table 7.3, Fig. 7.6). Only three central genes are significantly different between the two organisms and these could encode specific properties for enzymes assembling the repeating unit. Analysis of the structure of stewartan (Nimtz *et al.*, 1996b) revealed a replacement of the central galactose residue in the backbone of amylovoran by a glucose residue, a substitution of the pyruvate in the side-chain by a terminal glucose (see Fig. 7.2) and the lack of any substitutions such as acetyl, succinyl or pyruval groups. Amylovoran carries acetyl groups on the galactose of the side-chain (Nimtz *et al.*, 1996a). An enzyme for acetyl transfer is apparently not encoded

ams cluster of *E. amylovora*



cps cluster of *E. stewartii*

Fig. 7.6. Comparison of the *ams* gene clusters of *Erwinia amylovora* and *cps* gene clusters of *Erwinia stewartii*. The genes *amsB*, C, D, E and *cpsD*, E, F, G, located in the centre of the operons, are peculiar in their relationship to each other (see Table 7.3). Although *amsA* has high similarity to *cpsC*, *amsB* has the highest to *cpsE* and not to *cpsD*, adjacent to *cpsC*. Also the similarities of *amsC*, D and E do not correspond to the order of the *cps* genes. These genes seem to be involved in different tasks for the assembly of the central sugar residues of the backbone and the terminal residue in the side-chain of amylovoran and stewartan, respectively. The latter step may be catalysed by *AmsB/CpsE*, the former by *AmsD+E/CpsF+G* (see Figs 7.2 and 7.3).

Table 7.3. Comparison of the genes encoded in the *ams* region of *Erwinia amylovora* and the *cps* region of *Erwinia stewartii* for their amino acid and the nucleotide sequences. The values represent the percentage of identical residues, except for amino acids marked by a superscript a. Divergent genes are printed in bold.

<i>ams</i>	G	H	I	A	B	C	D	E	F	J	K	L	M
(size: aa)	(477)	(377)	(144)	(726)	(301)	(375)	(351)	(266)	(743)	(415)	(407)	(388)	(298)
<i>cps</i>	A	B	I	C	E	D	G	F	H	J	K	L	M
(size: aa)	(476)	(376)	(144)	(725)	(305)	(378)	(344)	(252)	(736)	(420)	(407)	(390)	(298)
Identical aa	77	81	69	74	74	37	32	18	60	70	73	76	80
(similar aa ^a)	(86)	(89)	(81)	(83)	(81)	(54)	(46)	(36)	(72)	(80)	(80)	(85)	(86)
Identical nt	72	75	71	74	69	55 ^b	47 ^b	60 ^b	62	68	71	73	73

^a Comparison including conservative changes.
^b Only local patches with intermediate homology (other values mostly comprise areas with high homology (> 75%)).
aa, Amino acids; nt, nucleotides.

by the *ams* operon, since transfer of the gene cluster into an *E. stewartii cps* mutant resulted in the synthesis of non-acetylated amylovoran (Bernhard *et al.*, 1996). The lack of substitution terminating the side-chain, but also the low level of hypersensitive reaction (HR) induction of *E. stewartii* could explain why stewartan synthesis does not result in ooze production on immature pears, although the heterologously complemented *E. stewartii cps* mutants were virulent on maize seedlings.

EPS of bacteria apart from the genus *Erwinia* and role in bacterial virulence

Many phytopathogenic bacteria produce exopolysaccharides. Aspects of their role in the bacterial interaction with plants have been the subject of two recent reviews (Leigh and Coplin, 1992; Denny, 1995).

Clinical problems can be caused by *P. aeruginosa*, which produces alginate. When the pathogen colonizes the lungs of patients suffering from cystic fibrosis, the severity of health problems is correlated with the amount of EPS synthesized (Govan and Deretic, 1996). Alginate consists of a polymer of mannuronate and guluronate, which is generated by epimerization of polymeric mannuronic acid residues. The *alg* gene cluster in *P. aeruginosa* is normally silent, but environmental factors, such as osmotic stress, low nutrients or lack of water, activate its expression (Roychoudhury *et al.*, 1992). Transcription is controlled by a two-component signal transduction, comprising phosphorylation of regulatory proteins. Some plant-pathogenic bacteria also produce alginate. The biosynthetic genes for alginate production are apparently highly homologous among pseudomonads. An alginate-deficient mutant of *Pseudomonas syringae* pv. *syringae* was constructed by insertion of a transposon in the gene *algL* (Peñaloza-Vázquez *et al.*, 1997). This deficiency in alginate synthesis did not result in lack of symptom formation on pear plantlets. The loss of alginate production could be complemented by *alg* genes of *P. syringae* pv. *syringae*, but not with the same genes from *P. aeruginosa*. Fusions with a reporter gene (*uidA*) showed a high level of transcription of the *algD* promoter at 32°C and stimulation of transcription by copper sulphate, sodium chloride and sorbitol. Copper ions also induced alginate synthesis in several other *P. syringae* pathovars (Kidambi *et al.*, 1995).

Succinoglycan is the major EPS of the symbiotic, nitrogen-fixing bacterium (*Sino*-)*Rhizobium meliloti* and is required for the bacterial nodule invasion. Succinoglycan-deficient mutants are apparently exposed to plant defence reactions. The structure of the repeating unit has been determined (see Fig. 7.3) and its biosynthesis has been largely elucidated by biochemical analysis of EDTA-treated cells (Reuber and Walker, 1993). The synthetic genes are located on one of two megaplasmids (Long *et al.*, 1988). Megaplasmid 1 carries nodulation and fixation genes and megaplasmid 2 carries 19 *exo* genes, involved in rhizobial EPS synthesis. Their order is not correlated to sequential steps of the gene

products in succinoglycan biosynthesis. The synthesis of the repeating unit is initiated by addition of a galactose to the lipid carrier, catalysed by ExoY and ExoF and the transfer of the subsequent sugars by ExoA, L, M, O, U and W. ExoV adds the pyruvate to the terminal glucose of the side-chain and ExoP, Q and T function in the transport or polymerization of the repeating unit.

Agrobacterium tumefaciens produces a succinoglycan, like *R. meliloti*. No particular phenotype could be correlated with *A. tumefaciens* mutants deficient in succinoglycan synthesis, in contrast to mutants in the *att* gene, which are deficient in a cell-associated acidic polysaccharide (Reuhs *et al.*, 1997). Strains unable to synthesize cellulose are reduced in attachment to plant cells and impaired in crown-gall tumour formation (Minnemeyer *et al.*, 1991). Two operons required for cellulose synthesis have been partially characterized (Matthysse *et al.*, 1995a) and a subcellular system has been established from *A. tumefaciens* for the biosynthesis of cellulose (Matthysse *et al.*, 1995b).

For several bacterial species, the role of EPS in the host plant–pathogen interaction could not be defined under laboratory conditions. Xanthan (see Fig. 7.3) is a commercially important exopolysaccharide, produced in huge amounts by *X. campestris*, and is added to many food products. Consequently, data about the genes for xanthan biosynthesis and their functions have been kept secret because of commercial interest (Leigh and Coplin, 1992). As often seen for vascular pathogens, no striking effect was observed for xanthan-deficient mutants in symptom formation (Denny, 1995). A similar situation has occurred for EPS-deficient mutants of *Ralstonia solanacearum*. These strains seem to be impaired in the entry to plants via the root system, and especially in causing wilting symptoms on host plants. Systemic colonization is similar for EPS producing and non-EPS producing strains (Denny, 1995). Colanic acid is the type I capsular polysaccharide of *E. coli* (see Fig. 7.3). Serologically, it is classified as an M antigen and is only produced in minimal medium and at low growth temperatures. Production of colanic acid is strongly increased in mutants that do not produce the *lon* protease. Due to an extended life time of RcsA in *lon* mutants, many steps in the regulation of colanic acid synthesis could be described for *E. coli* (Gottesman, 1995) and will be discussed in a later paragraph, together with regulation of EPS synthesis in *E. amylovora*.

Sugar metabolism and precursor synthesis

Activated sugar molecules are the basis of polysaccharide synthesis. Levan synthesis depends on the energy released from the hydrolysis of sucrose, where glucose is liberated and fructose polymerized. The formation of amylovoran depends on UDP-sugars, i.e. UDP-galactose, UDP-glucose and UDP-glucuronic acid. They have to be synthesized within the cells from the pool of highly energetic compounds, such as ATP or UTP.

Two genes at the end of the *ams* operon are involved in UDP-sugar precursor synthesis. The *galE* gene with galactose-epimerase activity is located

separately from the *gal* operon (Metzger *et al.*, 1994). Its expression is not induced by galactose. Its gene product is responsible for the formation of UDP-galactose (Fig. 7.7). AmsM is a GalF homologue and is also related to GalU. The gene product of *amsM* does not display a UDP-glucose pyrophosphorylase activity. Like GalF in *E. coli* (Marolda and Valvano, 1996), it may interact with GalU and modulate the pyrophosphorylase (Langlotz *et al.*, 1999).

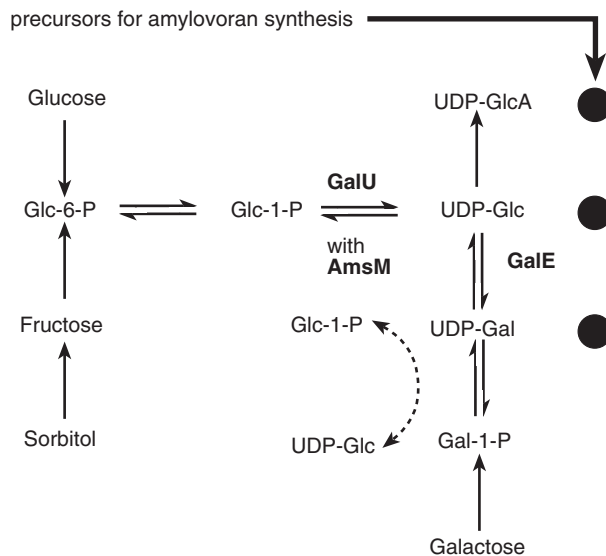


Fig. 7.7. UDP-sugar metabolism for precursor synthesis of amylovoran. The energy is supplied by the natural environment of *Erwinia amylovora* from carbohydrates indicated at the left side of the figure. UDP-glucose is also required for the conversion of galactose-1-phosphate to UDP-galactose.

General phosphorylation of HPr by soluble, cytoplasmic enzyme I:

P-enolpyruvate + enzyme I (EI) \rightleftharpoons P-EI + pyruvate

P-EI + HPr \rightleftharpoons P-HPr + EI

Transphosphorylation of carbohydrate-specific carrier protein:

P-HPr + EIIA (or EIIA + EIIE) \rightleftharpoons P-EIIA + HPr

P-EIIA + EIIB \rightleftharpoons P-EIIB + EIIA

(membrane-bound, hydrophobic domain of enzyme II: EIIC)

P-EIIB + carbohydrate (from outside) \rightarrow EIIB + carbohydrate-P (**transport into cell**)

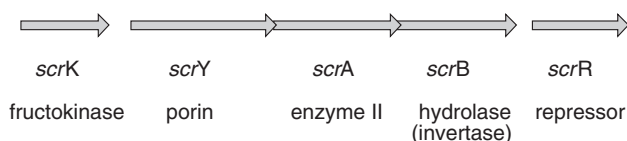
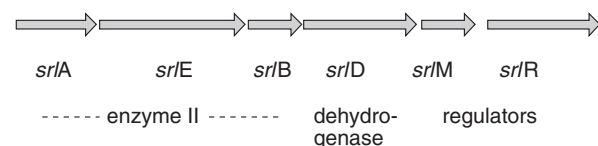
Fig. 7.8. Scheme for enzymes involved in carbohydrate uptake of bacteria. The phosphate donor is phosphoenol pyruvate and the energy is used for carbohydrate transport after a chain of phosphate transfers (Postma *et al.*, 1993).

have a high similarity (> 85%) with the N-terminal or C-terminal part of GutA of *E. coli* and should have a similar function to the *E. coli* enzyme (Yamada and Saier, 1987; Postma *et al.*, 1993).

In *E. amylovora*, the genes for sorbitol metabolism are clustered (Fig. 7.9). *E. amylovora* mutants in the sorbitol metabolism do not grow on minimal agar with sorbitol as the only carbon source. Gene *srlD*, encoding the dehydrogenase, is absolutely required for symptom formation on apple seedlings, whereas the *srlA/srlE* function may be partially substituted by other sugar uptake systems of the cell. The *srl* operon of *E. amylovora* is induced by sorbitol and repressed by glucose. The sugar alcohol sorbitol is intimately connected with fire blight symptoms (Suleman and Steiner, 1994) and can change in concentration during daytime and seasons in various parts of the plant tissue (Sakai, 1966; Chong and Tapper, 1971). Sorbitol is a good carbon source for *E. amylovora* to synthesize amylovoran (Bennett and Billing, 1978) and increases EPS synthesis (Bellemann *et al.*, 1994). Sorbitol could be a prerequisite for *E. amylovora* to colonize plants, restricting fire blight to members of the *Rosaceae*.

Sucrose is an important storage and transport sugar of all plants. Many, if not all, plant-associated bacteria are therefore able to metabolize this disaccharide. A DNA fragment with the sucrose operon was identified in a genomic library (J. Bogs and K. Geider, unpublished) and further investigated in *E. coli*, which cannot use sucrose as a carbon source. By transposon mutagenesis and subcloning of DNA fragments and nucleotide sequencing, a region with the metabolic genes of *E. amylovora* was characterized. Mutants in the sucrose operon of *E. amylovora* have a similar phenotype as the *srl* mutants, they are weakly or not at all virulent on apple seedlings. The sucrose genes are also clustered in an operon (see Fig. 7.9). Five ORFs encode functions for the uptake and degradation of sucrose (J. Bogs and K. Geider, unpublished). The ORFs have high

***srl* operon (uptake and metabolism of sorbitol)**



***scr* operon (uptake and metabolism of sucrose)**

Fig. 7.9. Genetic maps of the sorbitol and the sucrose operon. Both operons are induced by the corresponding carbohydrate. The sucrose cleavage is encoded by *scrB* and has been shown in *Bacillus subtilis* (as the *sacB* gene) also to carry a fructose-polymerizing activity.

homology to the genes of other organisms involved in sucrose metabolism. In contrast to the *lsc* gene, the *scr* operon is induced by sucrose. This was demonstrated by fusions with reporter genes and assays of sucrose hydrolase activity, encoded by *scrB* (J. Bogs and K. Geider, unpublished). When *gfp* was fused to the promoter region in front of *scrY*, and the regulatory gene *scrR* was over-expressed in *E. amylovora*, a response to sucrose in the environment was detected by flow cytometry.

Levansucrase and natural levan-deficient *E. amylovora* strains

Levan is synthesized by *E. amylovora* via the secreted enzyme levansucrase. In contrast to the soft-rot erwinias, *E. amylovora* releases only a few enzymes. Besides levansucrase, two secreted proteases were described (Seemüller and Beer, 1977). We have mutated the gene of the protease secreted in minimal medium and found no significant influence on virulence (Zhang *et al.*, 1999). Mutation of the *lsc* gene causes a retarded colonization of shoots by *E. amylovora*, whereas ooze formation on immature pears is normal (Geier and Geider, 1993). The enzyme was purified to homogeneity on sodium dodecyl sulphate (SDS) gels and gave two bands in isoelectric focusing (IEF) gels,

presumably due to different degrees of phosphorylation. Gene expression is not affected by the sugar composition of the medium. The constitutive expression may not be favourable in all situations during spread of fire blight.

In The Netherlands, a series of *E. amylovora* strains, isolated from orchards, were deficient in levan synthesis. Sequencing of the promoter regions in front of the *lsc* genes gave no indication for an alteration in their nucleotide sequence. When the genes were cloned by PCR amplification and inserted into a high-copy-number plasmid, levansucrase was expressed even in the mutant strains after reintroduction of their own *lsc* genes (Bereswill *et al.*, 1997). The analysis of residual activities of levan metabolism revealed two types of mutants. A strain of type 1 synthesized a normal amount of *lsc*-specific mRNA and was impaired in a later step, possibly translocation or secretion of the protein. Type 2 mutants did not transcribe the *lsc* gene, when assayed by Northern blots. They are apparently downregulated in transcription. A DNA fragment was cloned carrying the gene *rlsA*, which could complement the deficiency, and mutations in that gene inserted into the chromosome of a wild-type strain produced the levan-deficient phenotype (Zhang and Geider, 1999). The low level of levan synthesis was not changed by growth in various media. However, when the cultures were grown at 18°C instead of 28°C, expression of *lsc* was considerably increased in most of the deficient strains (Bereswill *et al.*, 1997). Apparently, their *lsc* genes are controlled by a temperature-sensitive regulatory system. *rlsA* was localized in the *hrp* region of *E. amylovora*, close to *dspA/B* (E/F) (Kim and Beer, Chapter 8) and may be controlled by the *hrp* system (Zhang and Geider, 1999).

Regulation of EPS synthesis by *E. amylovora*

The importance of continuous EPS synthesis by *E. amylovora* is emphasized by a sugar-independent expression of levansucrase (Geier and Geider, 1993), but also by a continuous expression of the *ams* operon from log-phase cells into the stationary growth phase (Bellemann *et al.*, 1994). Most genes of the *ams* region are transcribed as an operon, which could be shown as a 16 kb mRNA in a Northern blot (Bugert and Geider, 1995). Amylovoran synthesis is strongly affected by the genes *rcaA* (Bernhard *et al.*, 1990; Chatterjee *et al.*, 1990; Coleman *et al.*, 1990) and *rcaB* (Bereswill and Geider, 1997). Analogous genes were characterized in *E. coli* as activators of colanic acid synthesis (Gottesman, 1995). They belong to a two-component regulatory system, which is proposed to consist of the sensor RcsC, to transfer environmental signals to RcsB, mediated by attachment of a phosphate group. RcsA is supposed to bind to RcsB, converting it to an efficient activator. RcsA is also controlling the expression of *E. coli* K antigens (Keenleyside *et al.*, 1992). Its level in *E. coli* (Gottesman, 1995) or *E. amylovora* (Eastgate *et al.*, 1995) is affected by the *lon* protease, which belongs to the heat-shock proteins. Mutants in *rcaA* and *rcaB* produce little amylovoran. RcsB has been shown to bind to a DNA region in front of *amsG*, the first gene in the *ams* operon (Kelm *et al.*, 1997). This is consistent with the start

of the *ams* mRNA 103 nucleotides upstream of *amsG* (P. Bugert and K. Geider, unpublished) (Fig. 7.10).

These regulatory genes are related to other genes found in many Gram-negative bacteria. They are part of a network of regulatory events, which comprise DNA-binding proteins as well as additional regulators, such as RcsF (Gervais and Drapeau, 1992) or RcsV (Aldridge *et al.*, 1998). The gene *rcsV* is barely expressed in *E. amylovora*, in contrast to other erwinias. A viscous EPS is produced in *E. stewartii* and EPS synthesis is increased in *E. herbicola* and *E. ananas* after transfer of *rcsV* into these strains.

Expression of amylovoran synthesis depends on various environmental conditions, such as temperature, salt or carbon sources. To regulate its EPS production, a network of regulatory proteins seems to come into play, including global regulators, such as H-NS (P. Aldridge, M. Hildebrand and K. Geider, unpublished) or RsmA (Mukherjee *et al.*, 1996). The latter gene cross-hybridized with *csrA*, which affects glycogen synthesis in *E. coli* (Yang, H. *et al.*,

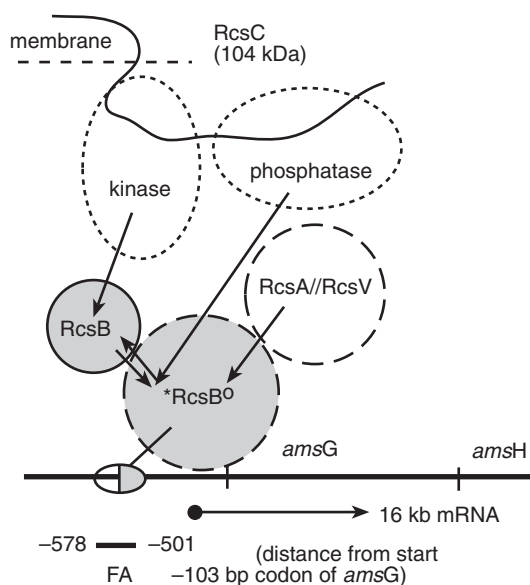


Fig. 7.10. Regulatory events controlling the *ams* region of *Erwinia amylovora* for EPS synthesis. The functions were also deduced from data obtained from Rcs proteins of *Escherichia coli*. FA, DNA fragment used for binding studies with *E. amylovora*-RcsB/RcsA (Kelm *et al.*, 1997). RcsC may have a dual role to interfere with RcsB, activation by phosphorylation and suppression by dephosphorylation. RcsB is further activated by RcsA. Since the RcsA-like protein RcsV is barely expressed in *E. amylovora* under assay conditions, its role for RcsB activation is less defined. It is open to question whether the activators RcsB/RcsA predominantly bind at the start region of the *ams* operon and also to internal sequences.

1996). Levan synthesis is independent of RcsA and RcsB, since its level in the corresponding mutants is not changed (Bereswill and Geider, 1997).

Conclusion

EPS produced by *E. amylovora* have multiple roles during colonization of host plants by this pathogen. Although amylovan is not tightly associated with the cells (Politis and Goodman, 1980), the loose capsule could modulate the action of the HR-inducing harpin (Kim and Beer, Chapter 8). This is functionally similar to its binding to lectins in order to prevent bacterial aggregation. The low affinity of *E. amylovora* for plant cells in contrast to *A. tumefaciens* may support plant colonization of EPS-embedded bacteria. Clogging of vessels could be a late event during migration in the xylem, when the bacterial density increases in already affected vascular bundles. This may cause extrusion into the parenchyma or, alternatively, bacteria lyse vessel cells by secretion of protease (Zhang *et al.*, 1999). Capsulated bacteria survive better under dry conditions, compared with uncapsulated cells (C. Langlotz and K. Geider, unpublished). This can be either a direct protection by an amylovan coat or preservation of residual water for the cell population in the presence of amylovan. The large amylovan is sensitive to processing. No data have been reported about whether its viscosity is affected by salt conditions of the environment. In addition to amylovan and levan, *E. amylovora* produces a low-molecular-weight glucan (Smith *et al.*, 1995). Its role could be stabilization of the fragile cell structure of *E. amylovora*, especially during osmotic changes, as described for other bacteria (Weissborn *et al.*, 1992). So far, glucan could support the function of the capsular EPS amylovan, which may also provide protection against changing salt concentrations or extreme loss of water during dry environmental conditions.

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***hrp* Genes and Harpins of *Erwinia amylovora*: a Decade of Discovery**

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Introduction: *Erwinia amylovora*, a death agent in the orchard

E. amylovora causes the devastating fire blight disease of rosaceous plants. In general, the disease affects apple and pear, as well as cotoneaster and other ornamental plants; strains isolated from these plant species are genetically homogeneous (Pérombelon, 1992; Momol and Aldwinckle, Chapter 4). Some strains in the USA and Canada, however, infect *Rubus* species plants but not apple and pear (Starr *et al.*, 1951). The genus *Erwinia* belongs to the family Enterobacteriaceae (Lelliott, 1984), which contains well-studied bacteria of medical importance, such as *Escherichia*, *Salmonella*, *Shigella* and *Yersinia* species. *Erwinias* are quite attractive for studying the mechanisms of host–pathogen interactions. Like their enteric cousins, erwinias are easy to culture and to study microbiologically. In fact, classic genetic analyses of *E. amylovora* and *Erwinia chrysanthemi* were extensively pursued in the 1970s (Chatterjee and Starr, 1980). Although the new genera *Pantoea* and *Pectobacterium* have been proposed for some *Erwinia* species (Gavini *et al.*, 1989; Hauben *et al.*, 1998), the name *Erwinia* will be retained in this review.

Soon after the bacterial aetiology of fire blight became clear near the end of the 19th century, questions on the mechanisms of the disease began to be pursued. However, it was only during the 1980s that meaningful answers began to appear. Molecular techniques initially developed for *Escherichia coli* were adapted to the study of *E. amylovora* (for example, Bauer, 1990). As a result, two factors, *hrp/dsp* genes and extracellular polysaccharides, were found to be important in pathogenesis by *E. amylovora* (Steinberger and Beer, 1988; Barny *et al.*, 1990; Vanneste *et al.*, 1990; Bellemann and Geider, 1992). This chapter describes the

initial discovery of *hrp* genes in *E. amylovora* and recent progress in our understanding of the roles of *hrp* genes in *E. amylovora* pathogenesis. Reviews on exopolysaccharides and *dsp* (disease-specific) genes of *E. amylovora* can be found elsewhere in this volume (Geider, Chapter 7; Bogdanove *et al.*, Chapter 9).

Discovery and early studies of *hrp* genes

Identification of *hrp* genes in phytopathogenic bacteria

Many Gram-negative plant-pathogenic bacteria elicit the defensive hypersensitive reaction (HR) in non-host plants (Klement, 1982; Goodman and Novacky, 1994). The macroscopic HR can be easily observed, usually 18–24 h after infiltration of high concentrations of bacteria ($\geq 5 \times 10^6$ cells ml⁻¹) into the intercellular spaces of plant leaves. During the HR, rapid K⁺/H⁺ exchange occurs, and rapidly dying plant cells release toxic compounds, which provide a harsh environment for the invading microbes. Early experiments on the bacteria-elicited HR established that a single bacterium can induce the HR of a single plant cell (Turner and Novacky, 1974) and that contact between bacteria and plant cells is critical for the development of an HR (Holliday *et al.*, 1981).

During the mid-1980s, it became evident that the same factors that control the ability to induce an HR in non-hosts control the ability to infect hosts. Genes were identified in *Pseudomonas syringae* that are absolutely required for both the HR and pathogenicity; hence they were called *hrp* (Lindgren *et al.*, 1986). Soon, *hrp* genes were also found in species of *Erwinia*, *Ralstonia* (a new genus set up for a group of *Pseudomonas* spp., including *Pseudomonas solanacearum* (Yabuuchi *et al.*, 1995)), and *Xanthomonas* (Willis *et al.*, 1991; Lindgren, 1997). Today, based on Southern hybridization, common regulatory genes, complementation assays and organization and sequence similarity of secretion genes, *hrp* genes can be divided into two groups: group I, containing genes of *Erwinia* and *P. syringae*, and group II, containing those of *Ralstonia solanacearum* and *Xanthomonas* (Alfano and Collmer, 1996).

Early research on *hrp* genes of *E. amylovora*

Clustered *hrp* genes of *E. amylovora* were first identified by transposon mutagenesis of strains Ea321 and Ea322 in the USA (Bauer and Beer, 1987, 1991; Steinberger and Beer, 1988). Subsequently, Ea321 DNA was cloned into a cosmid vector, pCPP9, to construct a genomic library; complementation of all previously obtained Hrp mutants by cosmid pCPP430 led to the conclusion that this cosmid harbours the entire *hrp* gene cluster (Beer *et al.*, 1989) (Fig. 8.1). Importantly, pCPP430 or either of two other *hrp* cluster-containing cosmids called pCPP440 and pCPP450 enabled recipient *E. coli* and other non-plant pathogens to elicit the HR in an array of plants like wild-type *E. amylovora* (Beer

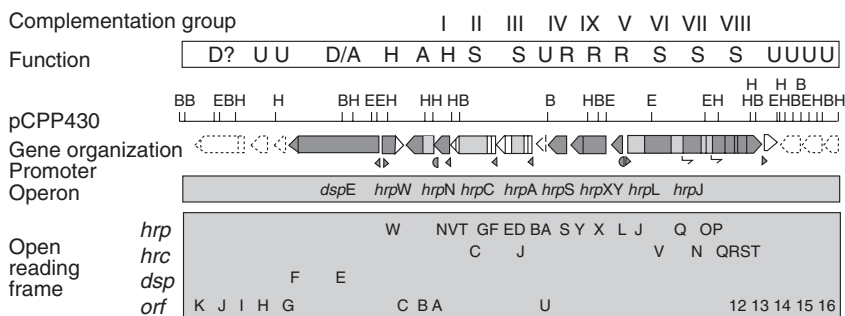


Fig. 8.1. Genetic arrangement and implicated functions of the *hrp* gene cluster of *Erwinia amylovora* Ea321 carried in cosmid pCPP430. Abbreviations in the function row: D, disease-specific; A, avirulence; H, harpin; S, secretion; R, regulation; U, unknown. In the gene organization row, directions of arrow boxes indicate the orientation of transcription, and regulatory *hrp* genes, *hrc* secretory genes, harpins and homologues of *avr* genes are stippled. Putative HrpL-dependent promoters are indicated by small triangles, σ^{54} promoters are indicated by half-circles and potential internal promoters are indicated by half-arrows.

et al., 1991). This development made it easier to dissect and analyse *hrp* genes of *E. amylovora* and greatly accelerated the characterization and understanding of the *E. amylovora* *hrp* system. These initial experiments indicated that genes required for the Hrp phenotype of *E. amylovora* are localized in a c. 20–25-kb region of DNA. Further analysis of the *hrp* gene cluster of *E. amylovora* suggested that it consists of eight complementation groups (see Fig. 8.1), which are involved in either production or secretion of an HR-elicitor protein called harpin (Wei and Beer, 1993).

A similar transposon mutagenesis approach was taken for strain CFBP1430 in France (Barney *et al.*, 1990; Vanneste *et al.*, 1990) and *hrp* DNA of this strain was cloned in several plasmids. Barney and colleagues also localized several mutants that are affected only in pathogenicity to a c. 5-kb region next to the *hrp* region (Bogdanove *et al.*, Chapter 9). In the UK, another group of scientists identified an *hrp* locus, which we believe is located in the *hrp* gene cluster, by complementing a non-pathogenic isolate with a genomic library of a virulent isolate (Walters *et al.*, 1990). More recently, two cosmids containing a functional *hrp* gene cluster of the *Rubus*-pathogenic strain Ea246 were isolated (Laby, 1997). Cross-complementation of Hrp⁻ mutants of Ea321 (an apple strain) with *hrp* DNA of Ea246 (a *Rubus* strain) indicated that *hrp* gene function is unlikely to be the basis for the host specificity of the two strains (Laby, 1997).

In addition to *E. amylovora*, most species in the genus *Erwinia*, including *Erwinia stewartii*, *Erwinia carotovora*, *E. chrysanthemi* and *Erwinia herbicola* pv. *gypsophylae*, have been demonstrated to contain *hrp* genes (Coplin *et al.*, 1992; Laby and Beer, 1992; Bauer *et al.*, 1994; Cui *et al.*, 1996; Nizan *et al.*, 1997).

HR elicitation by macerogenic bacteria and oncogenic bacteria is not usually observed under normal assay conditions (Klement, 1982). Thus, the finding of *hrp* genes in soft-rot and gall-forming erwinias was particularly surprising and suggested that the *hrp* system is a basic component of pathogenesis in the erwinias. Limited comparisons of *hrp* gene sequences among erwinias suggest that the *E. amylovora* *hrp* genes are more closely related to those of *Pantoea*-group species and less so to those of soft-rotting species. Thus, the phylogenetic relationships among species of *Erwinia* based on *hrp* genes is consistent with that suggested by comparison of 16S rDNA (Hauben *et al.*, 1998).

Functions of *hrp* gene products

The products of *E. amylovora* *hrp* genes can be classified into three categories based on their functions in Hrp pathogenesis: regulatory, secretory and secreted. Regulatory proteins control the expression of other *hrp* genes; secretory proteins, many of them structural components of a protein secretion apparatus, are involved in secreting target proteins to the cell exterior; and secreted proteins are delivered through the apparatus. Secreted proteins include harpins and potential effector proteins, which are likely to directly affect host metabolism and promote parasitism.

Complex regulatory cascades of *hrp* gene expression

In *E. amylovora*, expression of *hrp* genes is activated *in planta*, but repressed in rich media, such as Luria medium or nutrient medium; gene induction in culture occurs only under conditions that simulate the conditions of plant apoplasts. The environmental signals that affect gene expression include carbon and nitrogen sources, pH, temperature and osmolarity (Wei *et al.*, 1992b) (Fig. 8.2). Several minimal media that mimic the apoplastic environment have now been developed (Huynh *et al.*, 1989; Wei *et al.*, 1992b; Wengelnik *et al.*, 1996).

HrpL seems to be the master switch of the *hrp* systems of *E. amylovora* and *P. syringae* (Xiao *et al.*, 1994; Wei and Beer, 1995) (see Fig. 8.2). It belongs to a subfamily of eubacterial σ factors that regulate extracytoplasmic functions (Lonetto *et al.*, 1994), and it activates all secretory *hrp* operons, harpin genes and *dsp/avr* genes (Xiao and Hutcheson, 1994; Wei and Beer, 1995; Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998b; Kim and Beer, 1998). HrpL recognizes a conserved sequence motif, referred to as the '*hrp* box' (Innes *et al.*, 1993; Shen and Keen, 1993), located at the promoter regions of the HrpL-dependent operons or genes (Xiao and Hutcheson, 1994). Several *Erwinia* species contain similar motifs upstream of *hrp* genes or related genes (Table 8.1).

hrpL expression in *Erwinia* spp. appears to depend on the σ^{54} /HrpS system (Frederick *et al.*, 1993; Wei and Beer, 1995) (see Fig. 8.2). σ^{54} Consensus

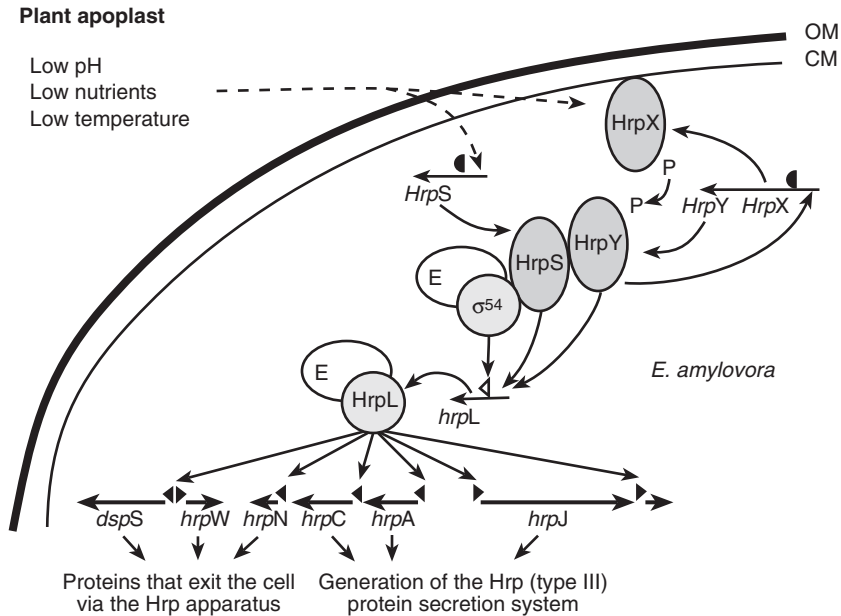


Fig. 8.2. Model for the regulation of *hrp* gene expression in *Erwinia amylovora*. HrpX/HrpY are the two-component regulatory proteins, HrpS is a σ^{54} enhancer-binding protein, and HrpL is an alternative σ factor. Thick arrow lines indicate genes or operons, ovals indicate proteins and arrowheads in thinner lines indicate the directions of information flow. CM, cytoplasmic membrane; OM, outer membrane; P, phosphate; E, RNA polymerase; closed half-circle, $\sigma 70$ promoter; open triangle, σ^{54} promoter; filled triangle, HrpL promoter.

sequences have been found from the promoter regions of *E. amylovora* and *P. syringae* *hrpL* (Xiao *et al.*, 1994; Z. Wei, J.F. Kim and S.V. Beer, unpublished), and *rpoN*, which codes for σ^{54} , is required for activation of an *E. stewartii* *hrp* locus (Frederick *et al.*, 1993) and *P. syringae* *hrpL* (Xiao *et al.*, 1994). In *E. amylovora*, expression of *hrpL* is partially controlled by HrpS (Wei and Beer, 1995), which is a member of the NtrC family of σ^{54} enhancer-binding proteins (Grimm and Panopoulos, 1989; Sneath *et al.*, 1990; Frederick *et al.*, 1993; Xiao *et al.*, 1994). While *E. amylovora* and *E. stewartii* contain only *hrpS* (Frederick *et al.*, 1993; Z. Wei, J.F. Kim and S.V. Beer, unpublished), *P. syringae* contains *hrpS* and a remarkably similar regulatory gene, *hrpR* (Grimm and Panopoulos, 1989; Xiao *et al.*, 1994; Grimm *et al.*, 1995). Two different models have been proposed for the regulation of *P. syringae* *hrpL* by HrpR and HrpS (Xiao *et al.*, 1994; Grimm *et al.*, 1995). Some genes in the *E. amylovora* *hrp* gene cluster are preceded by potential σ^{54} promoters, suggesting that they are controlled by the σ^{54} /HrpS system, rather than by HrpL (J.F. Kim and S.V. Beer, unpublished data).

Table 8.1. Conserved motifs found in the promoter regions of genes or operons of *Erwinia* species that are regulated by HrpL or suspected to be, and consensus sequence of the HrpL-dependent promoter of *Pseudomonas syringae*.

Organism	Gene or operon	Conserved sequences and consensus	Location ^b	Reference or source
<i>E. amylovora</i>	<i>hrp/dsp</i>	nn GGAA AC Y nnnnnnnnnnnnnn CCAC T YAAW nnnnnnn -35 -10 +1		
	<i>hrpA</i>	TGGGAACCGATCGAA ACTGCCCGCCACTTAATTAAACGA	62	Kim <i>et al.</i> (1997)
	<i>hrpC</i>	ACGGAACCTCGCCACGCGCCGAAACCCCACTCAAAAGACAGG	37	Kim <i>et al.</i> (1997)
	<i>hrpI</i>	AGGGAACCGATGGCT CAATCGCACACACAATGACAAAC	39	Bogdanove <i>et al.</i> (1996b)
	<i>hrpN</i>	CCGGAACCGAGCGG AATAACGAGACTCAATATAAGC	80	Wei and Beer (1995)
	<i>hrpW</i>	GCGGAACCCCTGTCAA CGCAAACCCACTCAATTCAGGC	63	Kim and Beer (1998)
	<i>dspE</i>	TGGGAACCGGTTGCA GAGAAATTGCAACATAAAAATATC	46	Bogdanove <i>et al.</i> (1998b)
	<i>orf12</i>	ACGGAACCTATTACCT GCCGTTCCGCACCTATTCCTCCGA	35	Bogdanove <i>et al.</i> (1996b)
	<i>hsvG</i>	CCGGAACCGCCGGGC GGTTTTTCGTTACAAAAAGAGGGAG	68	Valinsky <i>et al.</i> (1998)
<i>E. herbicola</i> ^c	<i>wt5E</i>	GTGGAACTATCCACG CAAACTCACACACAAAAGATAAT	51	D.L. Coplin, personal communication
<i>E. carotovora</i>	<i>hrpN</i>	TGGGAACCTGAGCAGG CAAGAAAAATCACTTAAATGGGGGA	84	Mukherjee <i>et al.</i> (1997)
<i>E. chrysanthemi</i>	<i>hrpC</i>	ATGGAAACCGCCGCCA CTCCCCGGCCACACAACTGCAGG	66	Kim <i>et al.</i> (1998)
	<i>hrpN</i>	GAGGAACCGGTTTCAC CGTCGGCGTCACTCAGTAAACAAG	101	Bauer <i>et al.</i> (1995)
<i>P. syringae</i>	<i>hrp/avr</i>	nt GGAA CCnannnnnnnnnnnnnn CA CnnAnnnnnnnn		Xiao and Hutcheson (1994)

^a Conserved nucleotides at the -35 and -10 regions and potential transcription initiation sites are underlined. Characterized transcription start sites are doubly underlined. Bold-faced nucleotides are consensus sequences. Y, C or T; W, A or T; n, any base.

^b Number of nucleotides from the last conserved C at the -10 region to the start codon.

^c *hsvG* gene is present in plant-pathogenic strains pv. *betae* and pv. *gypsophilae*.

In *E. amylovora*, two other regulatory proteins, HrpX and HrpY, activate expression of *hrpL* (Z. Wei, J.F. Kim and S.V. Beer, unpublished) (see Fig. 8.2). They are members of the two-component regulatory protein family that is widely used for prokaryotic gene expression. HrpX is a putative sensor that probably perceives environmental signals, and HrpY is a potential accompanying response regulator, which may transmit the signal from HrpX to *hrpL*. *hrpY* is absolutely required for the Hrp phenotype and *hrpL* expression, whereas *hrpX* seems partially involved in Hrp function (Z. Wei, J.F. Kim and S.V. Beer, unpublished). Although *hrpX* has a high basal level of gene expression, its expression is induced under *hrp*-inducing conditions.

Other factors also appear to be involved in the regulation of *hrp* genes. In *E. carotovora* subsp. *carotovora*, *hrpN* is regulated by homoserine lactone and global regulators, RsmA and *rsmB* (for regulator of secondary metabolites) (Cui *et al.*, 1996; Mukherjee *et al.*, 1997; Liu *et al.*, 1998). The *hrp* clusters of *E. amylovora* and *P. syringae* contain a gene, *hrpJ*, which is a homologue of *yopN* of *Yersinia* spp. (Bogdanove *et al.*, 1996b). YopN is a type III secreted protein thought to act as a 'contact sensor' (Rosqvist *et al.*, 1994). Although their sequence similarity is rather low (22% identical), homology between HrpJ and YopN suggests that polarized transfer of *E. amylovora* effector proteins may also be regulated in a contact-dependent manner.

Hrp protein secretion pathway

Initial sequence analysis in the early 1990s surprisingly indicated homology between several Hrp proteins and proteins of animal-pathogenic bacteria that are involved in the secretion of virulence proteins (Van Gijsegem *et al.*, 1993). To distinguish this conserved secretion system from the haemolysin secretion and general secretory pathways, the system was designated the 'type III' secretion pathway (Salmond and Reeves, 1993; for a recent comprehensive review, see Hueck, 1998), which is commonly referred to as the Hrp pathway for plant pathogens.

By the mid-1990s, confusion in *hrp* gene names increased as the number of *hrp* genes identified from different bacteria increased. To clarify relationships among homologous *hrp* genes in different organisms, the name *hrc* (for HR and conserved) (Bogdanove *et al.*, 1996a) was given to nine highly conserved *hrp* secretory genes. Except for *hrcC*, homologues of *hrc* genes are also found in the flagellar biogenesis systems of eubacteria. Sequence analysis, comparison with homologues and experimental evidence suggest that *hrc* genes encode one outer-membrane protein (HrcC), one lipoprotein (HrcJ), five polytopic inner-membrane proteins (HrcR, HrcS, HrcT, HrcU, HrcV) and one cytoplasmic ATPase homologue (HrcN) (Bogdanove *et al.*, 1996a) (Fig. 8.3). The subcellular location of HrcQ is unclear, but a homologue in *Salmonella enterica*, SpaO, is exported via the type III pathway (Li *et al.*, 1995). Other less conserved Hrp secretory proteins include HrpQ of *E. amylovora* and *P. syringae* and HrpW of *R.*

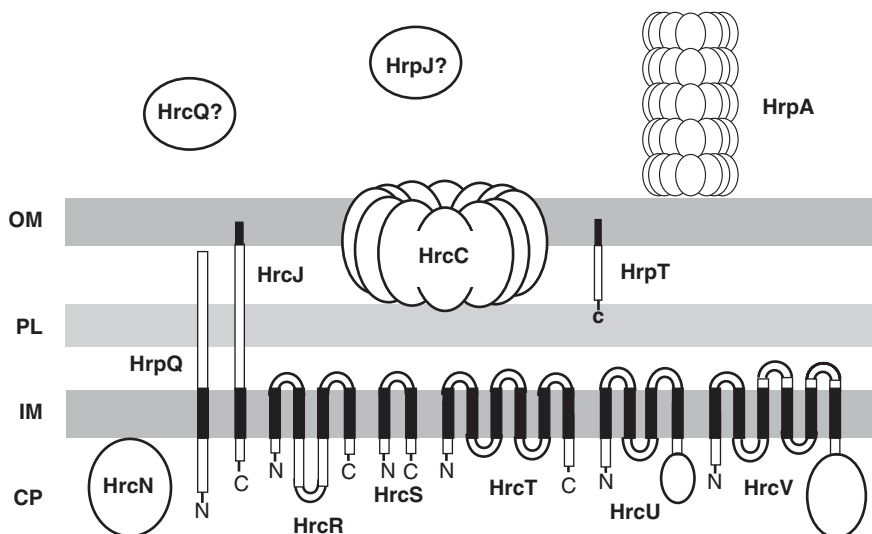


Fig. 8.3. Predicted locations and conformations of Hrp/c proteins of *Erwinia amylovora* based on sequence analysis and information from homologues. Secretory Hrp/c proteins are thought to form a complex across the bacterial membranes. HrpA is a component of the Hrp pilus. Homologues of HrcC form multimers. HrcJ and HrpT are putative lipoproteins (the acylated N terminus is indicated by a thick line). The HrpQ homologue in the SPI-1 type III system of *Salmonella typhimurium* is a component of the basal-body-like structure. Black boxes in Hrc proteins and HrpQ are putative membrane-spanning domains. The N-terminal regions of HrcV homologues are predicted to contain six to eight transmembrane domains. OM, outer membrane; PL, peptidoglycan layer; IM, inner membrane; CP, cytoplasm.

solanacearum, which are homologous to *Yersinia* YscD (Bogdanove *et al.*, 1996b), and HrpE of *E. amylovora* and *P. syringae*, HrpF of *R. solanacearum*, and HrpB5 of *Xanthomonas campestris* pv. *vesicatoria*, which are homologous to YscL/FliH (Kim *et al.*, 1997). HrpQ homologues in *Shigella flexneri*, *Yersinia pestis* and enterohaemorrhagic *E. coli* have been localized to the membrane fractions (Allaoui *et al.*, 1995; Plano and Straley, 1995; Kresse *et al.*, 1998), and one homologue in the *S. enterica* SPI-1 system, PrgH, is a component of the type III secretion structure (Kubori *et al.*, 1998).

Among the Hrc proteins, HrcC is worthy of special attention in that homologues are present in the type II secretion system (Pugsley, 1993), but not in flagellar export systems. Furthermore, HrcC homologues, such as PulD, OutD and pIV, form an outer-membrane protein complex called secretin, which functions as a 'gatekeeper' by specifically recognizing their target proteins (Russel, 1994). Also worth noting is the fact that HrcJ homologues in the type III systems are about half the size of their homologue (FliF) in flagellar systems. FliF is an inner-

membrane protein that forms the flagellar basal body (Macnab, 1996). Despite these and other differences between the type III system and the flagellar system, the supramolecular structure of the type III apparatus of *S. enterica* (Kubori *et al.*, 1998) (and possibly other type III systems) is surprisingly similar to the flagellar structure.

The number of bacteria known to possess the type III secretion system is escalating. During recent years, the presence of type III secretion genes was recognized in *Rhizobium* species (*nol/hrc*; Meinhardt *et al.*, 1993; Freiberg *et al.*, 1997), several pathogenic strains of *E. coli* (*sep/esc*; Jarvis *et al.*, 1995; Elliott *et al.*, 1998; Perna *et al.*, 1998), *Pseudomonas aeruginosa* (*psc/pcr*; Yahr *et al.*, 1996; Frank, 1997), and even *Chlamydia* species (Hsia *et al.*, 1997; Stephens *et al.*, 1998). Interestingly, *Salmonella* employs two different type III systems to enter host cells and survive inside them (Ochman *et al.*, 1996; Shea *et al.*, 1996). Thus, the type III system is cosmopolitan among important pathogens of animals and plants, and study of the *E. amylovora* Hrp pathway could have a broad impact on pathogenic microbiology.

Travellers of the Hrp pathway

Harpins: Hrp-secreted HR-eliciting proteins

Harpins elicit the HR in plants and were the first proteins demonstrated to be secreted via the Hrp pathway (Wei *et al.*, 1992a; He *et al.*, 1993; Wei and Beer, 1993; Arlat *et al.*, 1994). They are hydrophilic (acidic), glycine-rich and lack cysteine. *E. amylovora* has two harpin genes, *hrpN* and *hrpW*, which are located at one end of the *hrp* gene cluster (Wei *et al.*, 1992a; Kim and Beer, 1998) (see Fig. 8.1). Although harpins have been identified based on their ability to elicit the HR when infiltrated into intercellular spaces of plants (Alfano and Collmer, 1996), their biological function in pathogenesis remains unclear. *hrpN* mutants of *E. amylovora* Ea321 almost completely lose HR-eliciting activity and pathogenicity (Wei *et al.*, 1992a; Kim and Beer, 1998). Mutations in the same gene of *E. amylovora* CFBP1430 (Barney, 1995) and *E. chrysanthemi* (Bauer *et al.*, 1995), however, only partially reduce these activities. Furthermore, *hrpW* mutants of *E. amylovora* (Kim and Beer, 1998), *hrpN* mutants of *E. stewartii* (D.L. Coplin, personal communication), and *hrpW* or *hrpZ* mutants of *P. syringae* (Alfano *et al.*, 1996; Charkowski *et al.*, 1998) are not affected in HR elicitation or pathogenicity.

It is enigmatic how harpins, apparently heterogeneous in sequence and possibly different in structure, can elicit similar plant reactions. The 'cell-killing' action of harpins does not appear to be due to potential enzymatic or toxic function; rather, the HR-eliciting activity is heat-stable and requires plant metabolism, and fragments of harpins can elicit the response (Alfano and Collmer, 1996; Laby, 1997; Charkowski *et al.*, 1998; Kim and Beer, 1998). Thus, harpins

may act as signalling molecules that induce programmed cell death (Greenberg, 1996). Avr proteins also elicit the HR in plants carrying corresponding resistance genes (Staskawicz *et al.*, 1995). It seems unlikely, however, that plants recognize harpins and Avr proteins similarly, although downstream signalling events that lead to the HR might be shared. Possible signalling mechanisms that lead to the HR against harpins were discussed by Novacky and colleagues (Hoyos *et al.*, 1996).

Harpins appear to act on the outer parts of plant cells. They elicit the HR when exogenously applied to plant tissue by infiltration. When harpins are added to cell-suspension culture, K⁺ efflux and alkalinization of the medium occurs, followed by cell death (Wei *et al.*, 1992a; Popham *et al.*, 1995). However, this rapid ion-exchange reaction, which is called the XR, does not occur in protoplast culture (Hoyos *et al.*, 1996; Z. Wei and S.V. Beer, unpublished data). In addition, HrpZ antibodies have helped to localize the HrpZ protein outside plant cells and not in protoplasts. Furthermore, alkalinization of media and localization of the protein are blocked by a chelating agent that extracts Ca²⁺ and pectin (Hoyos *et al.*, 1996). The homology of HrpW to pectate lyases (Charkowski *et al.*, 1998; Kim and Beer, 1998) is consistent with a model in which the site of harpin action is the plant cell wall.

Harpins applied to plants have been shown to induce systemic resistance against pathogens (Strobel *et al.*, 1996; Wei and Beer, 1996; H. Dong, J.F. Kim and S.V. Beer, unpublished results) and to induce repellence against insects (Zitter and Beer, 1998). Recent studies indicate that HrpN treatment of *Arabidopsis* and tobacco plants induces pathogenesis-related proteins. Plants defective in the salicylic acid-dependent pathway, however, fail to develop systemic resistance in response to HrpN (Dong *et al.*, 1999), suggesting that harpin-induced resistance functions through this pathway. Interestingly, simple treatment, such as spraying leaf surfaces, drenching roots or soaking seeds, is sufficient for harpin activity (Qiu *et al.*, 1997). Also intriguingly, HrpN can promote the growth of tobacco and several other plants (Qiu *et al.*, 1997). The mechanisms underlying these phenomena are still unclear, but the beneficial effects of harpins are being actively pursued for potential use in agriculture (Mullin *et al.*, 1998; Page-Lester *et al.*, 1998; Schading *et al.*, 1998; Wei *et al.*, 1998). In addition, an approach to controlling plant diseases by expressing the *hrpN* gene in plants in a pathogen-inducible fashion is being developed on a number of plant species (S.V. Beer *et al.*, unpublished results).

Other Hrp-secreted proteins

In addition to harpins, several other proteins may be delivered via the Hrp pathway of *E. amylovora* (Table 8.2). A number of proteins have been detected in culture supernatants of *E. amylovora* Ea321 and Ea273, but not from their *hrp* secretion mutants (Kim, 1997; J.F. Kim and S.V. Beer, unpublished results). One

Table 8.2. Proteins that may travel the Hrp (type III) protein secretion system of *Erwinia amylovora*.

Protein	Class	Supporting evidence	Reference
HrpJ	Regulation	Homologue (YopN of <i>Yersinia</i> spp.) is exported via the type III pathway	Bogdanove <i>et al.</i> (1996b); Forsberg <i>et al.</i> (1991)
HrcQ	Secretion	Homologue (SpaO of <i>Salmonella typhimurium</i>) is exported via the type III pathway	Bogdanove <i>et al.</i> (1996b); Li <i>et al.</i> (1995)
HrpA	Hrp pilus	Homologue in <i>Pseudomonas syringae</i> is exported via the Hrp pathway	Kim <i>et al.</i> (1997); Roine <i>et al.</i> (1997)
HrpN	Harpin	Western analysis of culture supernatant using HrpN-specific antibodies	Wei <i>et al.</i> (1992a)
HrpW	Harpin	Western analysis of culture supernatant using HrpW-specific antibodies	Kim and Beer (1998)
DspE	Avr-like	Western analysis of culture supernatant using DspE-specific antibodies	Bogdanove <i>et al.</i> (1998a)
OrfB	Avr-like	Homologues (YopJ/YopP of <i>Yersinia</i> spp. and AvrA of <i>Salmonella enterica</i>) are secreted via the type III pathway	Kim (1997); Galyov <i>et al.</i> (1994); Mills <i>et al.</i> (1997); Hardt and Galan (1997)

of these is the pathogenicity protein DspE (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998a); OrfB might be another (Kim, 1997). DspE and OrfB are discussed in depth in the chapter on *dsp* genes (Bogdanove *et al.*, Chapter 9). Some structural proteins in the *hrp* secretory operons are also suspected to be Hrp-delivered, based on their homology with proteins of other type III systems (Kim *et al.*, 1997). For example, HrpJ is homologous to the regulatory protein YopN of *Yersinia* species, which is secreted via the type III pathway.

Like flagellar assembly systems (Macnab, 1996) and type III systems of animal pathogens (Ginocchio *et al.*, 1994; Parsot *et al.*, 1995), the Hrp system of *P. syringae* pv. *tomato* produces a surface appendage called the Hrp pilus (Roine *et al.*, 1997). *E. amylovora* may also form an Hrp pilus, since HrpA, a structural protein for the Hrp pilus (Roine *et al.*, 1997), has been detected in the *hrp*-induced culture supernatant of *E. amylovora* (J.F. Kim and S.V. Beer, unpublished results; S.Y. He, personal communication). The role of the Hrp pili may be to deliver effector proteins from the bacterial cytoplasm to plant cells or to provide for close contact between bacteria and plant cell, facilitating translocation of effector proteins.

The Hrp pathogenicity island

Depending on the bacterial species, *hrp* genes are located either on plasmids (*R. solanacearum* and *E. herbicola* pv. *gypsophilae*) or on the chromosome (*E. amylovora*, *P. syringae* and *Xanthomonas* spp.) (Bonas, 1994). They have not been

found in non-pathogenic relatives of plant pathogens, including several strains of *E. herbicola* and *Pseudomonas fluorescens* (Huang *et al.*, 1988; Laby and Beer, 1992). Recent analyses of the *hrp*-flanking regions of *E. amylovora* (J.F. Kim and S.V. Beer, in preparation) and *P. syringae* (J.R. Alfano, A.O. Charkowski and A. Collmer, in preparation) indicate the presence of tRNA genes and cryptic recombinase genes at one end. In *E. amylovora*, genes beyond these are very similar to those in *E. coli*; the same is true beyond the other end of the *hrp* gene cluster. Preliminary results suggest that the *hrp* gene cluster of *E. chrysanthemi* is also linked to a transposase gene (J.F. Kim, A. Collmer and S.V. Beer, unpublished data).

Bacteriophage and conjugative transposons often utilize highly conserved tRNA genes as a landmark to insert their DNA into the host chromosome. Also, in animal pathogens, genes encoding virulence factors are often located in discrete chromosomal segments, called 'pathogenicity islands' (Hacker *et al.*, 1997). The observations on the *hrp*-flanking regions of *E. amylovora* and *P. syringae* suggest that the *hrp* gene clusters of these bacteria are parts of a large pathogenicity cassette, which we call the 'Hrp pathogenicity island', and *hrp* genes may have been subject to horizontal transfer. The latter notion is supported by a discrepancy between bacterial and *hrp* gene phylogenies: although *Xanthomonas*, *Erwinia* and fluorescent *Pseudomonas* are taxonomically closer to each other than to *Ralstonia*, *hrp* genes in *Xanthomonas* and *Ralstonia* are closely related to each other and are distinct from those of *Erwinia* and *P. syringae* (Bogdanove *et al.*, 1996a).

Conclusion: future directions of *E. amylovora* *hrp* gene research

In summary, *hrp* and *hrp*-associated genes encode proteins that positively regulate other *hrp* genes, are assembled into a specialized protein secretion apparatus or are secreted from the bacterial cell (Fig. 8.4). The HR we observe seems to be the outcome of a collective effect of harpins and virulence proteins, which are delivered by the Hrp secretion mechanism to plant intercellular spaces or inside plant cells. These proteins may disturb the plant's metabolism in the susceptible host by attacking components of plant cells or disrupting signalling pathways. These bacterial virulence proteins may be perceived by non-hosts or hosts armed with R proteins that specifically recognize virulence proteins (virulence proteins then become avirulence proteins), resulting in HR development, precluding bacterial establishment and thwarting disease.

During the past 10 years, we have learned that major components of pathogenesis are shared between plant and animal pathogens, and have come to realize that they utilize a common strategy to colonize eukaryotic hosts. Although the accumulated information on the *hrp* system and Hrp-secreted proteins is daunting, we still have many more questions than answers. We do not know how *hrp* genes evolve, how they are regulated spatially and temporally, how the

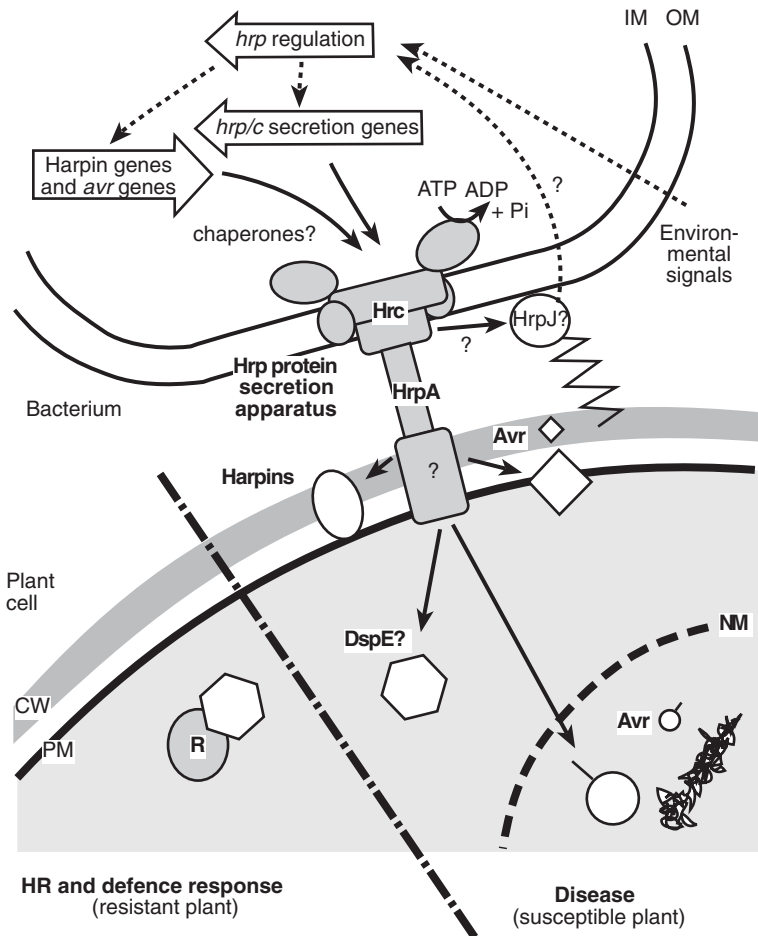


Fig. 8.4. Hypothetical model of the Hrp protein secretion apparatus and possible destinations of Hrp-transported proteins of *Erwinia amylovora*. Hrc proteins are thought to be core proteins that constitute the Hrp apparatus. HrpJ is a putative contact sensor that may be a part of the secretion complex. HrpA is a component of the Hrp pilus, which may form a conduit through which effector proteins are secreted or may make close contact between the bacterium and a plant cell. Harpins may function at the plant cell wall or may assist virulence effector proteins to get into plant cytoplasm or nucleus. Virulence proteins, including Ave-like proteins, may travel the secretion pathway to be destined for the plant targets. DspE is a Hrp-secreted pathogenicity protein of *E. amylovora* that is functionally homologous to AvrE of *Pseudomonas syringae*. IM, inner membrane; OM, outer membrane; CW, plant cell wall; PM, plant plasma membrane; NM, nuclear membrane; R, plant resistance gene.

secretion system works mechanistically, what the full tally of proteins that are secreted is, and what the precise functions of the secreted proteins are. The molecular basis of the host specificity of *E. amylovora* is yet another important question. Answers to these questions, we hope, will lead us toward an understanding of the basis of fire blight and other bacterial diseases of plants and animals, and ultimately toward applications of more effective controls of the diseases.

Summary

To infect its woody hosts, *E. amylovora* uses a set of clustered genes termed *hrp* (hypersensitive reaction and pathogenicity), which is located on an apparent pathogenicity island. Studies on the *E. amylovora hrp* system indicate that the components consist of three functional classes. Regulatory genes, located at the centre of the cluster, control the expression of other genes in the cluster. Secretory genes, many of them named *hrc*, produce proteins that are suggested to form a transmembrane protein secretion apparatus called the Hrp (or type III) pathway. Finally, several genes encode the proteins that travel the Hrp pathway, including harpins and potential effector proteins that contribute to proliferation of *E. amylovora* inside plants. Harpins, glycine-rich, heat-stable elicitors of the hypersensitive reaction, appear to be targeted to the plant cell wall. Interestingly, harpins applied to plants also induce systemic acquired resistance, confer insect resistance and promote plant growth, opening the possibility of utilizing harpins in agriculture. Other proteins may serve as triggers of plant defence as well as virulence.

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Disease-specific Genes of *Erwinia amylovora*: Keys to Understanding Pathogenesis and Potential Targets for Disease Control

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Introduction: discovery of a disease-specific (*dsp*) locus

During the 1970s and 1980s, new tools enabled phyto bacteriologists to begin to explore molecular mechanisms of bacterial plant pathogenicity. A variety of required genes from many Gram-negative bacterial species were quickly identified using transposon-mediated insertional mutagenesis. Some genes were found to be involved in toxin production (Gross, 1991), others in synthesis of extracellular polysaccharides (Leigh and Coplin, 1992). The largest class of new genes, however, were those required both for pathogenesis and for eliciting the hypersensitive reaction (HR) in non-host plants. These genes were designated *hrp* (for HR and pathogenicity) genes (Willis *et al.*, 1991; Kim and Beer, Chapter 8).

In *Erwinia amylovora*, transposon mutagenesis also revealed, along with *hrp* genes and *ams* genes (required for synthesis of the extracellular polysaccharide amylovoran (Bellemann and Geider, 1992; Geider, Chapter 7), genes that are required for pathogenicity but dispensable for HR elicitation (Steinberger and Beer, 1988; Barny *et al.*, 1990; Vanneste *et al.*, 1990; Bellemann and Geider, 1992). The disrupted genes were termed *dsp* by Barny and her colleagues (1990) to indicate their 'disease-specific' function (Boucher *et al.*, 1987). Barny *et al.* mapped transposon insertions in the mutants of strain CFBP1430 to a c. 5-kb region neighbouring the *hrp* gene cluster (Fig. 9.1). In a less virulent strain, Ea321, insertions that mapped to the same genetic location abolished pathogenicity and caused a reduction in HR elicitation (Wei *et al.*, 1992). Later,

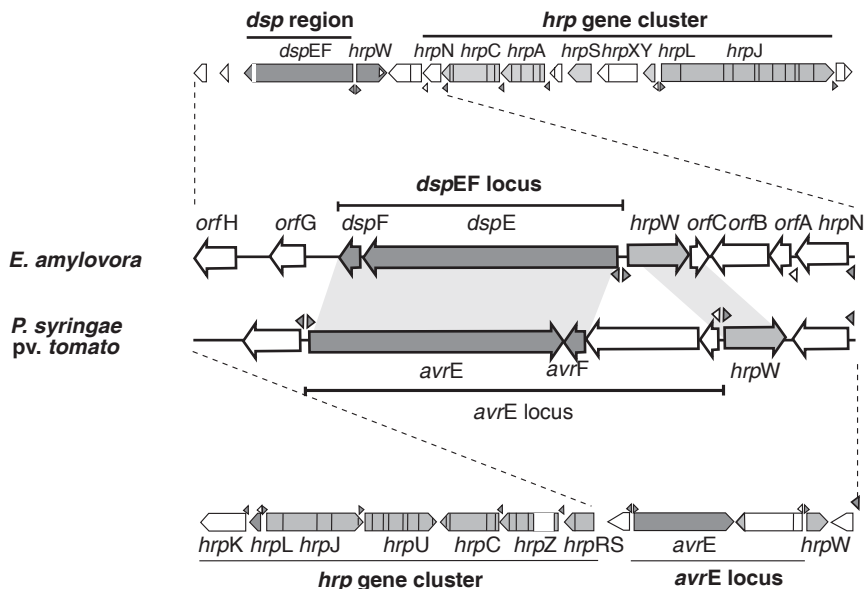


Fig. 9.1. The *dspEF* locus of *Erwinia amylovora* and its similarity with the *avrE* locus of *Pseudomonas syringae* pv. *tomato*. The *dsp* region is located at the *hrpN* end of the *E. amylovora* *hrp* gene cluster, separated by a region not required for the Hrp/Dsp phenotype. The *dspE* gene is homologous to the *avrE* gene of *P. syringae* pv. *tomato*, and *dspF* is homologous to *avrF*. Expression of *dspF* and *avrF* may be controlled differently from that of *dspE* and *avrE*, although the expression of *dspF* is enhanced when *dspE* expression is induced by HrpL. The *hrp* gene clusters of *E. amylovora* and *P. syringae* also show extensive similarities. Homologous operons of the two species are (respectively): *hrpC* and *hrpC*, *hrpA* and *hrpZ*, *hrpS* and *hrpRS*, *hrpL* and *hrpL*, *hrpJ* and *hrpJ/hrpU*. The *E. amylovora* *hrpN* gene is functionally analogous to *P. syringae* *hrpZ* in the *hrpZ* operon. The *hrp*–*dsp* connecting region contains another homologue of a *P. syringae* gene, *hrpW*. Homologous genes/operons are indicated by shading. Filled triangles denote HrpL-dependent promoters and open triangles indicate putative σ^{54} promoters. The map of the *P. syringae* *hrp* gene cluster is based on the *P. syringae* pv. *syringae* nucleotide sequence (Alfano and Collmer, 1997). See text for other references.

analysis of more Dsp[−] mutants suggested that, although most of the insertions are located at the *dsp* region, a few others are situated elsewhere in the genome, not linked to the *hrp*, *ams* or *dsp* region (Tharaud *et al.*, 1994). Also, some insertions that mapped to the *hrp* region resulted only in reduced virulence with no effect on HR elicitation and HR⁺.

Following the molecular cloning of the linked *hrp* and *dsp* regions in *E. amylovora* (Steinberger and Beer, 1988; Barny *et al.*, 1990; Beer *et al.*, 1991), work focused largely on the *hrp* region, resulting in many important and exciting findings (Kim and Beer, Chapter 8). More recently, work on the *dsp* region

and the *hrp*–*dsp* intervening region, including the determination of their complete nucleotide sequences, has been equally fruitful. The findings (Gaudriault *et al.*, 1997, 1998; Kim, 1997; Bogdanove *et al.*, 1998a, b; Kim and Beer, 1998) have not only shed light on the molecular basis of *E. amylovora* pathogenicity, but have also yielded surprising clues regarding the general nature and evolution of bacterial proteins that are involved in interactions with plants. Furthermore, information garnered so far points to some of these proteins as potential targets for novel means of fire blight control.

Genetic characterization of the *dsp* region

The *dsp* region is located next to one end of the *hrp* gene cluster, separated from the *hrpN* gene by *c.* 4 kb of DNA. The *dsp* region is comprised of a 6.6-kb apparent operon containing two genes (see Fig. 9.1). The region was characterized by Gaudriault *et al.* (1997) in France and by Bogdanove *et al.* (1998b) in the USA. Gaudriault and colleagues characterized the genes using strain CFPB1430 and named the genes *dspA* and *dspB*; Bogdanove and co-workers used strains Ea321 and Ea273 and the names *dspE* and *dspF*, based on their homology to genes in the *avrE* locus of *Pseudomonas syringae* pv. *tomato*. The nucleotide sequences of the genes from strains CFBP1430 and Ea321 are 100% identical. The designations of Bogdanove *et al.* will be used in this review.

dspE encodes a protein of 1838 amino acid residues, which is predicted to be hydrophilic and to have a basic isoelectric point (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998b). Based on SDS-PAGE, its apparent molecular weight is 190–200 kDa. *dspF* encodes a protein of 139 amino acid residues, which is predicted to be acidic and mostly α -helical (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998b). The apparent molecular weight of the DspF protein is 15.5 kDa. From these physical characteristics and because it is coexpressed with the DspE protein, DspF resembles chaperone proteins for virulence factors secreted through type III secretion pathways of animal-pathogenic bacteria, including species of *Salmonella*, *Shigella* and *Yersinia* (Wattiau *et al.*, 1996).

dspE is preceded by a sequence that matches the HrpL-dependent promoter consensus sequence, the so-called ‘*hrp* box’, of *E. amylovora* (Wei and Beer, 1995; Kim *et al.*, 1997) (see Fig. 9.1). HrpL is a putative alternate sigma factor required for the expression of most *hrp* genes in *P. syringae* and *E. amylovora* (Kim and Beer, Chapter 8). Expression of *dspE* is under the control of *hrpL* and resembles that of *hrp* genes with regard to response to environmental stimuli; namely, the gene is not expressed in rich media but is induced under low-nutrient conditions and *in planta* (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998b). The timing of *dspE* expression relative to *hrp* gene expression during the development of infection remains to be examined. Expression of *dspF*, which lies 61 bp downstream of *dspE*, is also increased by HrpL, presumably due to the promoter upstream of *dspE* (Gaudriault *et al.*, 1997). Unlike *dspE*, however, *dspF* is expressed constitutively at relatively high levels, apparently being driven by its own promoter.

Immediately downstream of *dspF* is A/T-rich DNA, followed by an open reading frame (ORF), similar to the *lysR* family of regulatory genes (Schell, 1993), which includes *spvR* of *Salmonella typhimurium* (Caldwell and Gulig, 1991) (see Fig. 9.1). The next downstream ORF is homologous to members of the Clp/Hsp100 family of ATPases (Wawrzynow *et al.*, 1996). Some insertions in these ORFs in Ea321 caused a reduction in virulence and HR-eliciting ability (S.V. Beer *et al.*, unpublished results). Whether this phenotype is due to an *hrp*-specific role for the putative genes or due to some role in general metabolism is not clear.

Additional transposon mutagenesis and in-frame deletion mutagenesis confirmed that both *dspE* and *dspF* (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998b) are required for fire blight symptoms and dispensable for HR elicitation. As suggested by the early transposon mutagenesis studies, Bogdanove *et al.* demonstrated with non-polar deletions that mutagenesis of *dspE* in a low-virulence strain Ea321 causes a reduction in HR-eliciting ability in tobacco leaves, which is not evident in *dspE* mutants of more virulent strains, CFBP1430 (Gaudriault *et al.*, 1997) and Ea273 (Bogdanove *et al.*, 1998b).

Homology of the *dspEF* locus with the *avrE* locus of *Pseudomonas syringae* pv. *tomato*

The deduced amino acid sequence of *dspE* (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998b) showed similarity to a partial sequence, deposited by Lorang *et al.* (Lorang and Keen, 1995), of the polygenic avirulence locus *avrE* of the bacterial speck pathogen, *P. syringae* pv. *tomato*. The entire *avrE* locus was sequenced and homologues of *dspE* and *dspF* containing 30% and 43% amino acid sequence identities, respectively, were found (Bogdanove *et al.*, 1998b). These homologues were named the *avrE* and *avrF* genes (Fig. 9.1). These genes are roughly the same size as the *E. amylovora* genes, with amino acid similarity distributed over their full lengths. The predicted products, AvrE and AvrF, also share physical characteristics with DspE and DspF, respectively. The locus comprises two convergent transcription units regulated by HrpL (Lorang and Keen, 1995) (see Fig. 9.1). The first contains the *avrE* gene (Bogdanove *et al.*, 1998b) and is preceded by an *hrp* box. The other contains *avrF* and uncharacterized genes upstream, and is preceded by a putative σ^{54} promoter.

Bacterial *avr* genes specifically limit host range by generating signals that elicit defence responses in some genotypes of host plants (Dangl, 1994; Leach and White, 1996). A corresponding resistance (R) gene in the plant is required for defence-response elicitation by a given *avr* gene (Staskawicz *et al.*, 1995). *avr* genes have traditionally been considered as negative determinants of host specificity at the race-cultivar level, but some, including the *avrE* locus, may restrict host range at the pathovar-species or species-species level (Whalen *et al.*, 1988; Kobayashi *et al.*, 1989; Swarup *et al.*, 1992). Many *avr* determinants, including *avrE*, are regulated by the *hrp* system (Leach and White, 1996). Interestingly,

avrE of *P. syringae* pv. *tomato* and *avrPphE* of *P. syringae* pv. *phaseolicola* (Mansfield *et al.*, 1994; Lorang and Keen, 1995) are physically linked to *hrp* genes. The *avrE* locus contributes quantitatively to the virulence in tomato leaves of *P. syringae* pv. *tomato* strain PT23, but is completely dispensable for full virulence of strain DC3000 (Lorang *et al.*, 1994; Lorang and Keen, 1995). Only a few other *avr* loci play detectable roles in pathogen fitness or in virulence in hosts tested (Kearney and Staskawicz, 1990; Swarup *et al.*, 1991; De Feyter *et al.*, 1993; Ashfield *et al.*, 1995; Ritter and Dangl, 1995). Thus, the selective force driving the maintenance in pathogen genomes of many of these host-range-limiting factors has remained a mystery for a long time.

Functional similarity of *dspEF* and *avrE* in virulence and avirulence

When expressed *in trans* on a plasmid, the *avrE* locus renders *P. syringae* pv. *glycinea*, which causes bacterial blight of soybean, avirulent in each of ten tested cultivars (Lorang and Keen, 1995). Just as the homologous *avrE* locus, the *dspEF* locus of *E. amylovora* introduced on a plasmid renders *P. syringae* pv. *glycinea* race 4 avirulent on soybean. The *avrE* locus of *P. syringae* pv. *tomato*, *in trans*, restores pathogenicity to *dspE* mutants of *E. amylovora* in immature pear fruits, although restored strains are low in virulence. Thus, the *dspEF* locus and the *avrE* locus function similarly and function transgenerically (Bogdanove *et al.*, 1998b). These findings clearly demonstrate a dual functionality for these loci, which may be typical of genes that encode Avr-like effector proteins of plant-pathogenic bacteria. Further, these data indicate that genetic background, in part, determines the relative contribution of homologous virulence/avirulence genes to disease. Therefore, the data suggest that many *avr* genes, for which no virulence phenotype has yet been detected, may have roles in disease.

Bogdanove *et al.* (1998b) suggested that the differences in genetic background between *E. amylovora* and *P. syringae* that lead to the dramatic difference in the virulence phenotypes of the homologous *dspEF* and *avrE* loci might be due to a quantitative or qualitative difference in the coevolution with plant hosts experienced by these pathogens. The authors propose that evolution in plants of corresponding *R* genes and modified targets of the factors encoded by the *dspEF* and *avrE* loci (and other virulence loci), which would lead over time to the modification or substitution of the factors (Alfano and Collmer, 1996), are likely to have occurred more in the numerous herbaceous hosts typically infected by *P. syringae* pathovars than in the relatively fewer and more slowly reproducing woody hosts with which *E. amylovora* presumably evolved. As an alternative explanation, they also suggest that *E. amylovora* might have evolved more recently than *P. syringae*, and thus had relatively less evolutionary time for proliferation and modification of virulence factors, which would lead to redundant function.

The homology and abilities of the *dspEF* and *avrE* loci to function

transgenerically also support the model proposed by Alfano and Collmer (1996), in which the proliferation of virulence factors that is driven by the counter-evolution of disarming mechanisms in plants (*R* genes and modified targets) can occur through the acquisition and modification of novel virulence factors from heterologous pathogens, and that such an 'evolutionary strategy' would require conservation of a functionally cosmopolitan virulence factor delivery system, such as the Hrp pathway (and possibly conservation of a universal Hrp-pathway-targeting signal on the factors themselves). The presence of *dspEF* and *avrE* in distinct genera suggests horizontal transfer of an ancestral locus, and, although *dspEF* and *avrE* are homologous and *hrp*-linked, the trans-generic function of these genes suggests that the Hrp pathways in *E. amylovora* and *P. syringae* have remained insensitive to differences accrued in DspE and AvrE over evolution. It seems likely that even non-homologous Avr-like proteins will function across phytopathogenic bacterial genera. Indeed, many *avr* genes are scattered in their distribution among pathogen strains and often carried on mobile elements (Dangl, 1994; Kim *et al.*, 1998), and, between *E. amylovora* and *P. syringae*, individual *hrp* genes are conserved (Bogdanove *et al.*, 1996; Kim *et al.*, 1997) and even functionally interchangeable (Laby and Beer, 1992) (see Fig. 9.1).

Hrp-dependent secretion of DspE and possible role of DspF as a chaperone

For interaction with plant cells, proteins produced by bacteria may be transported to the plant–bacterium interface or delivered to the plant cell interior. Gaudriault *et al.* (1997) showed that cell washes of *E. amylovora* grown on a solid minimal medium contain a protein corresponding in apparent molecular weight to DspE. Cell washes of both a *dspE* mutant and an Hrp pathway mutant did not contain the protein, providing an indication that DspE is an Hrp-secreted protein. Working independently, Bogdanove *et al.* (1998a) used an anti-DspE antiserum to examine cell and supernatant fractions of wild-type and appropriate mutant strains grown in an *hrp*-inducing minimal medium. By doing so, they demonstrated conclusively that *E. amylovora* secretes DspE via the *hrp*-encoded type III secretion pathway.

Gaudriault *et al.* (1997) also observed that the protein corresponding to DspE was absent from the cell washes of a *dspF* mutant. Taken together with the physical similarity of DspF to chaperons required for type III secretion of virulence factors from animal-pathogenic bacteria, the data provide evidence in favour of a role for DspF as molecular chaperone to DspE. Additional studies, including DspE–DspF interaction studies and subcellular localization of DspE, will be required to confirm this role. If confirmed, such a finding would be the first example of a chaperone required for secretion of an avirulence factor by phytopathogenic bacteria. In animal pathogens, the requirement of chaperones appears to be due to a role other than targeting to the secretion pathway

(Wattiau *et al.*, 1996): chaperones may stabilize proteins, maintain proteins in an appropriate conformation for secretion or prevent premature polymerization or association with other proteins. A chaperone might be important for DspE, due to its large size and probable multidomain nature (Bogdanove *et al.*, 1998a).

Implications of DspE secretion for its function and for delivery of Avr signals

Before DspE, the only proteins known to be secreted via the Hrp pathway were harpins and a structural component of the Hrp pilus (Kim and Beer, Chapter 8). A number of virulence proteins of animal-pathogenic bacteria, such as *Yersinia* spp., *Shigella flexneri*, *S. typhimurium* and pathogenic strains of *Escherichia coli*, are transported into host cells via type III pathways in a cell-contact-dependent manner (Hueck, 1998). To function, *avr* genes typically require the Hrp pathway (Leach and White, 1996). Indirect, but strong, evidence suggests that Avr proteins are similarly translocated to the plant cell interior via the Hrp pathway. Four *avr* genes, including *avrB* and *avrPto* of *P. syringae*, have been shown to elicit the hypersensitive response when expressed in plant cells (Van den Ackerveken and Bonas, 1997). Virulence proteins of animal-pathogenic bacteria that are transported into host cells are also secreted into the extracellular space under certain culture conditions (for a *Yersinia* example, see Cornelis, 1998). However, before the study on DspE (Bogdanove *et al.*, 1998a), which is a functional Avr protein, no natively expressed Avr protein had been reported outside bacterial cells in culture or *in planta*. Thus, *E. amylovora* yielded the first direct evidence that Avr proteins travel the Hrp (type III) pathway. The Hrp system of *Erwinia chrysanthemi* or *E. amylovora* cloned in laboratory strains of *E. coli* has since been shown not only to deliver signals of *P. syringae* *avrB* and *avrPto* to plants to elicit the R-gene-specific HR, but also to secrete AvrB and AvrPto proteins into the culture medium (Ham *et al.*, 1998; D.W. Bauer and S.V. Beer, unpublished results). Similarly, *E. amylovora* carrying *avrB* or *avrPto* secretes the avirulence proteins in culture (D.W. Bauer and S.V. Beer, unpublished results).

It has not been determined whether DspE is released into the apoplast (leaf intercellular space) *in planta*, which could indicate a plant extracellular or cell-surface-associated target in apple, pear and other host plants. Nor has it been determined whether DspE is translocated to the plant cell interior during attempted colonization by *E. amylovora*. Bogdanove *et al.* (1998a) found that a cell-free preparation of DspE (and DspF) failed to elicit the HR when infiltrated into soybean leaves, a finding consistent with other Avr proteins tested (Van den Ackerveken and Bonas, 1997) and with the possibility that the target, or receptor, of DspE in soybean is inside the plant cell. Nevertheless, definitive evidence awaits discovery. *In planta* subcellular localization of DspE will be an important step toward understanding both the virulence and avirulence functions of the *dspEF* locus.

The potential of a plant resistance protein recognizing DspE for control of fire blight

Monogenic (R-gene-mediated) resistance to fire blight has not been reported, but differential virulence of *E. amylovora* strains on apple cultivars has been observed (Norelli *et al.*, 1984). Also, some strains of *E. amylovora* infect *Rubus* spp. and not pomaceous plants, and vice versa (Starr *et al.*, 1951; Momol and Aldwinckle, Chapter 4). The *dspEF* operon is the first described locus in *E. amylovora* that may function in the avirulence of *E. amylovora*. We have also found in the region between the *hrp* and *dsp* loci of *E. amylovora* homologues of the *avrRxv* and *avrBsT* genes of tomato-pathogenic strains of *Xanthomonas campestris* pv. *vesicatoria* (Whalen *et al.*, 1993; Ciesiolka *et al.*, 1999) (see below). Like *avrE*, *avrRxv* introduced into other pathovars of *X. campestris* is responsible for resistance induction by the normally virulent strains in bean, soybean and several other plants (Whalen *et al.*, 1988). Also, *avrBsT* elicits the HR in all pepper lines tested, and loss of *avrBsT* allows a tomato strain of *X. campestris* pv. *vesicatoria* to cause disease in some of the normally resistant pepper lines (Minsavage *et al.*, 1990). Whether DspE, the AvrRxv/AvrBsT homologue, or other similar proteins indeed contribute to host specificity and differential virulence (avirulence) of *E. amylovora* remains to be determined.

Although the *dspEF* operon triggers defence responses in soybean when expressed in *P. syringae* pv. *glycinea*, it is not required for the HR of soybean elicited by *E. amylovora*, suggesting the presence of another elicitor-encoding gene in *E. amylovora* (Bogdanove *et al.*, 1998b). This could be HrpN or HrpW, two harpins *E. amylovora* secretes that elicit the HR in tobacco and many other species (Beer *et al.*, 1993; Kim and Beer, 1998). However, soybean appears to be insensitive to these proteins applied exogenously (Bogdanove *et al.*, 1998b; Kim and Beer, 1998). This fact suggests that the elicitor might be the AvrRxv/AvrBsT homologue or another Avr-like gene product recognized by an R protein in soybean.

Recognition of DspE in soybean suggests the possibility of engineering resistance in hosts of *E. amylovora* by using DspE to isolate a corresponding R gene from soybean (or another, more genetically tractable, non-host plant) and then transferring the gene to the host species. R-gene-mediated resistance to the apple scab pathogen *Venturia inaequalis* (Williams and Kuc, 1969) and successful transformation of apple with attacin E for control of fire blight (Norelli *et al.*, 1994; Norelli and Aldwinckle, Chapter 14) attest to the feasibility of such an approach. R-gene-mediated resistance to apple scab has been overcome in the field (Parisi *et al.*, 1993), but the requirement for *dspE* in disease favours the relative durability of a corresponding R gene (Dangl, 1994). Avirulence screening of *dspE* and other *E. amylovora* genes in well-studied relatives of the pathogen's hosts could broaden the pool of candidate R genes and hasten their isolation. Recently, this approach was undertaken with *Arabidopsis* to isolate an R gene specific to *dspE*. *Arabidopsis* ecotypes that develop a *dspE*-specific HR have been identified, and the gene(s) responsible for this phenotype are now being mapped

(E.R. Garr, D.W. Bauer and S.V. Beer, unpublished results). A similar approach could be used to screen for heterologous *R* genes effective against other pathogens of woody plants. Also, if the *dspEF* operon is as widely conserved as is suggested by its homology with the *avrE* locus, a corresponding *R* gene could be effective against a variety of pathogens of both woody and herbaceous plants.

Dsp and other Avr-like proteins in *E. amylovora* and other erwinias

The *E. amylovora dspEF* locus is located adjacent to the *hrp* gene cluster, connected by a region not important for the Hrp or Dsp phenotype (Gaudriault *et al.*, 1997; Kim, 1997). Sequence analysis of this region in strains Ea321 and Ea246 identified four ORFs, designated *orfA*, *orfB*, *orfC* and *hrpW* (Kim, 1997; Kim and Beer, 1998; J.F. Kim, R.J. Laby and S.V. Beer, unpublished data) (see Fig. 9.1). This gene organization appears to be conserved in CFBP1430 (Gaudriault *et al.*, 1998).

orfA and *orfB* are apparently in the same transcriptional unit and *orfA* is preceded by a putative σ^{54} promoter (Kim, 1997). The OrfA protein is homologous to members of the SycH family of chaperones (Wattiau *et al.*, 1996), and OrfB is a homologue of AvrRxx/AvrBsT of *X. campestris* pv. *vesicatoria* (Whalen *et al.*, 1993; Ciesiolka *et al.*, 1999), Y41O of *Rhizobium* sp. NGR234 (Freiberg *et al.*, 1997), AvrA of *Salmonella enterica* (Hardt and Galan, 1997) and the secreted protein YopJ/YopP of *Yersinia* spp. (Galyov *et al.*, 1994; Mills *et al.*, 1997). These homologies suggest that OrfB may be an Avr effector protein secreted via the type III pathway, and that OrfA may be its chaperone. Recently, homologues of *orfA* and *orfB* were also identified in *P. syringae* pv. *syringae* B728a (A.O. Charkowski and A. Collmer, unpublished results). Other erwinias are very likely to contain these genes as well.

Two interesting genes involved in host specificity come from studies of gall-inducing *Erwinia herbicola* strains. *E. herbicola* pv. *betae* (*Ehb*) infects beet and gypsophila, whereas *E. herbicola* pv. *gypsophilae* (*Ehg*) infects only gypsophila. Pathogenicity of these strains depends on a c. 150-kb plasmid called pPATH, which contains *hrp* genes (Nizan *et al.*, 1997). Valinsky *et al.* (1998) found a gene on pPATH, *hsvG* (for host-specific virulence), which is flanked by two insertion sequence elements and is required for the pathogenicity of *Ehb* on gypsophila but not on beet. The same group also identified from the plasmid a virulence gene of *Ehg* that, when introduced *in trans*, causes *Ehb* to be avirulent on beet (Barash *et al.*, 1998).

Gall-inducing pathovars of *E. herbicola* also contain a plasmid-encoded pathogenicity locus that is homologous to *dspEF* (I. Barash, personal communication). Homologues of *E. amylovora dspE* and *dspF* have been identified in *Erwinia stewartii* as well (Table 9.1), in which they are required for induction of water-soaking and wilt on maize (D.L. Coplin, personal communication). The

Table 9.1. Genes of *Erwinia* species with disease-specific phenotypes and those that may function in host specificity.

Organism	Gene(s)	Features	Reference
<i>E. amylovora</i>	<i>dspEF</i>	Pathogenicity factor; functionally similar to the <i>avrE</i> locus of <i>Pseudomonas syringae</i> pv. <i>tomato</i>	Bogdanove <i>et al.</i> (1998b)
	<i>orfAB</i>	<i>orfB</i> is homologous to <i>avrRxv</i> and <i>avrBsT</i> of <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	Kim (1997)
<i>E. herbicola</i> ^a	<i>dspEF</i>	Pathogenicity factor; homologue of <i>E. amylovora dspEF</i>	I. Barash, personal communication
	<i>hsvG</i>	Host-specific virulence factor; flanked by two insertion sequence elements	Valinsky <i>et al.</i> (1998)
	ORF	ORF of pv. <i>gypsophilae</i> expressed in pv. <i>betae</i> incites HR on beet	Barash <i>et al.</i> (1998)
<i>E. stewartii</i>	<i>wtsEF</i>	Required for wilt induction and water-soaking on maize; homologue of <i>dspEF</i>	D.L. Coplin, personal communication

^a *E. herbicola* pathovars *gypsophilae* and *betae*.

requirement for these homologues in this variety of *Erwinia*-caused diseases suggests that the *dspEF* gene family may be fundamental to pathogenesis by members of the genus as a whole.

A final word

The transgeneric conservation of the *dspEF* gene family, as well as its requirement for the development of fire blight, make it an attractive target for continued study. Elucidation of the virulence and avirulence functions of DspE and DspF will contribute greatly to our understanding of the molecular basis of the pathogenicity of *E. amylovora* and other erwinias. Further study also promises to open the door to the development of novel and effective means of control of fire blight, as well as other diseases caused by *Erwinia* and *Pseudomonas* species.

Summary

An *E. amylovora* locus required for pathogenesis in host plants but not required for elicitation of defence responses in non-host plants has recently been characterized. This 'disease-specific' (*dsp*) locus contains two genes, *dspE* and *dspF*, and flanks the *hrp* gene cluster. The *dspEF* locus is homologous with the *avrE* locus of *P. syringae* and functions as an avirulence locus when expressed in a pathovar of *P. syringae* that infects soybean. The *dspE* gene product travels the Hrp secretion pathway, apparently in a *dspF*-dependent manner. A number of other genes with potentially related functions in host specificity and pathogenicity have also been discovered in *E. amylovora* and in other *Erwinia* spp. Findings to date carry far-reaching implications regarding the nature and evolution of bacterial effector proteins involved in plant pathogenesis. The broad physical and functional conservation of the *dspEF* locus renders it a key target for strategies to better understand and to control fire blight and other bacterial diseases of plants.

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Iron and Fire Blight: Role in Pathogenicity of Desferrioxamine E, the Main Siderophore of *Erwinia amylovora*

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Introduction

Infectious diseases are the result of the interaction between a host organism and a pathogen. In this competitive relationship, the pathogen expresses virulence factors that enable it to defeat host defence reactions. Production of siderophores is one of the various weapons developed by the pathogen. These compounds provide an efficient strategy; not only do they allow the pathogens to overcome conditions of iron limitation encountered in host tissues, but they may also act as protective agents against iron toxicity. The release of siderophores is essential to the virulence of many animal and human pathogens (for a review, see Payne, 1993). For plant pathogens, iron has been shown to be critical in the soft-rot disease incited by *Erwinia chrysanthemi* strain 3937 on African violets; the control of the intracellular iron pool in relation to environmental conditions is central to the pathogenicity of this bacterium (for a review, see Expert *et al.*, 1996). Fire blight caused by *Erwinia amylovora* provides another interesting siderophore-related subject, since extracellular development of the pathogen and absence of rapid host cell death may lead to iron competition between the two protagonists. Furthermore, since several strains of *E. amylovora* produce siderophores belonging to the class of desferrioxamines (DFO) (Feistner *et al.*, 1993; Kachadourian *et al.*, 1996), it is possible that these siderophores, because of their structural properties (see next section), could be involved in pathogenicity in other ways than strictly contributing to bacterial iron acquisition. This review discusses the involvement of DFO in the development

of fire blight, and focuses on traits of siderophore structure that are important to consider when investigating the potential physiological role of these compounds. Several aspects concerning the biochemistry and molecular genetics of the *E. amylovora* DFO-dependent iron transport pathway are being studied in different laboratories. Recent advances on this topic, although still fragmentary today, are presented. Finally, possibilities for technical exploitation of the basic knowledge of the *E. amylovora* high-affinity iron transport pathway in terms of disease control are discussed.

Siderophore structure and iron chelation

'Siderophores are beautifully designed for the chelation and transport of iron(III)' (Hider, 1984). Siderophores are low-molecular-weight compounds (< 1500 Da) possessing a high affinity for Fe(III), which is the predominant form of iron in aerobic and microaerobic environments. Formation constants of ferrisiderophores ($K_f = [\text{Fe-Sid}] : [\text{Fe}] [\text{Sid}]$) are higher than 10^{20} . Produced by many microorganisms, siderophores were first identified as growth factors, before being characterized as iron chelators and iron transporters necessary for solubilizing and supplying this essential metal to the cell. Biosynthesis of siderophores, as well as of cognate proteins allowing transport across the bacterial membrane of the ferrisiderophores, are regulated by soluble iron levels present in the environment.

The structure of more than 100 siderophores has already been determined. Siderophore structure is usually determined by a variety of spectrometric methods, including nuclear magnetic resonance and mass spectrometry, along with chemical degradation and synthesis. When possible, single-crystal X-ray diffraction is used, as in the case of ferrioxamine E, for which good-quality crystals can be obtained (van der Helm and Poling, 1976). The originality of siderophores resides in their capability of both binding iron with high affinity and specificity and releasing it appropriately inside the cell. Determination of the thermodynamic and kinetic characteristics of siderophore-iron(III) complexes is particularly useful for the physiologist to predict and explain specific effects mediated by siderophores. Furthermore, studies of the mechanics of siderophore have a great interest for the synthesis of artificial analogues reproducing the properties of natural compounds (Bullen and Griffiths, 1987; Shanzer and Libman, 1991; Hider *et al.*, 1992). Iron chelators are required for the treatment of diseases associated with the abnormal distribution of metals, such as iron overload and lack of iron.

The ferric ion forms stable bonds with ligands containing weakly polarizable atoms, such as oxygen. Of the wide range of oxygen ligands, hydroxamate (e.g. desferrichrome and desferrioxamine) and catecholate (e.g. enterobactin and chrysobactin) (Figs. 10.1 and 10.2) have been selectively chosen by microorganisms for the formation of specific and stable complexes with the ferric iron (for reviews, see Neilands and Leong, 1986; Winkelmann *et al.*, 1987). Oxygen is not

the exclusive atom linked to iron, as illustrated by agrobactin, where coordination to an oxazoline nitrogen has been demonstrated (see Fig. 10.1). An α -hydroxy-carboxylic centre occurs in a number of siderophores, derived from citrate, such as aerobactin (see Fig. 10.1). Recently, a new class of siderophores has been found to contain only aliphatic amines and/or carboxylate and hydroxy donor groups (Thieken and Winkelmann, 1993). Achromobactin, a siderophore produced by *E. chrysanthemi*, in addition to chrysobactin, probably belongs to this latter class (Kachadourian, 1996). Hexadentate siderophores, such as trihydroxamates and catecholates, are ideal iron scavengers, because they are kinetically and thermodynamically stable. These ligands release the bound metal via reduction. The resulting iron(II), kinetically and thermodynamically much less stable than

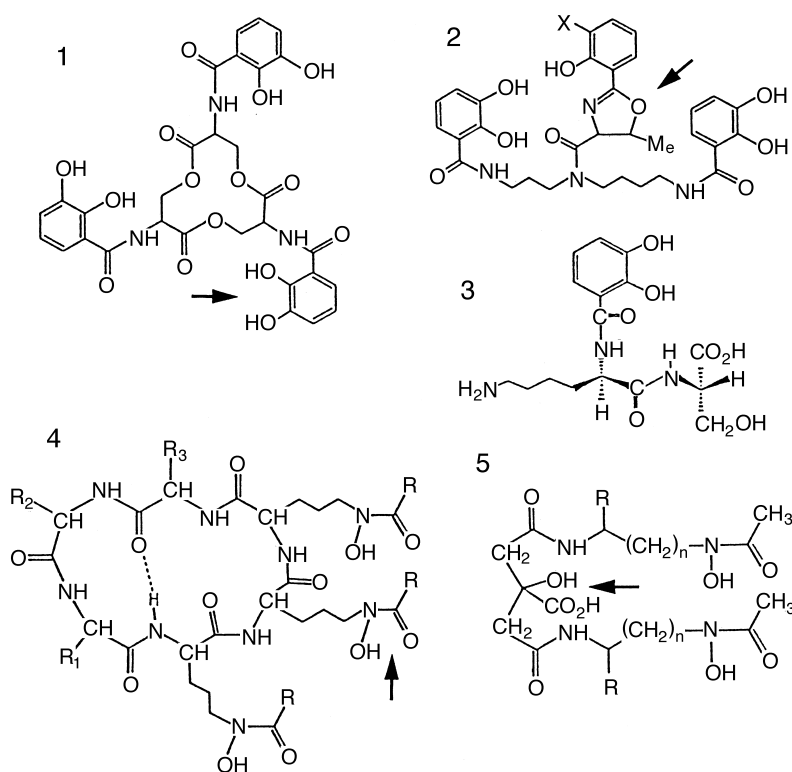


Fig. 10.1. Structure of some microbial siderophores: enterobactin or enterochelin (1), agrobactin (X = OH) (2) and chrysobactin (3) contain catechol ligands. In addition, agrobactin contains an oxazoline ring. Desferrichrome (4) and aerobactin (5) are hydroxamates. Aerobactin also contains a hydroxycarboxylate ligand. Iron(III) ligands are shown by arrows.

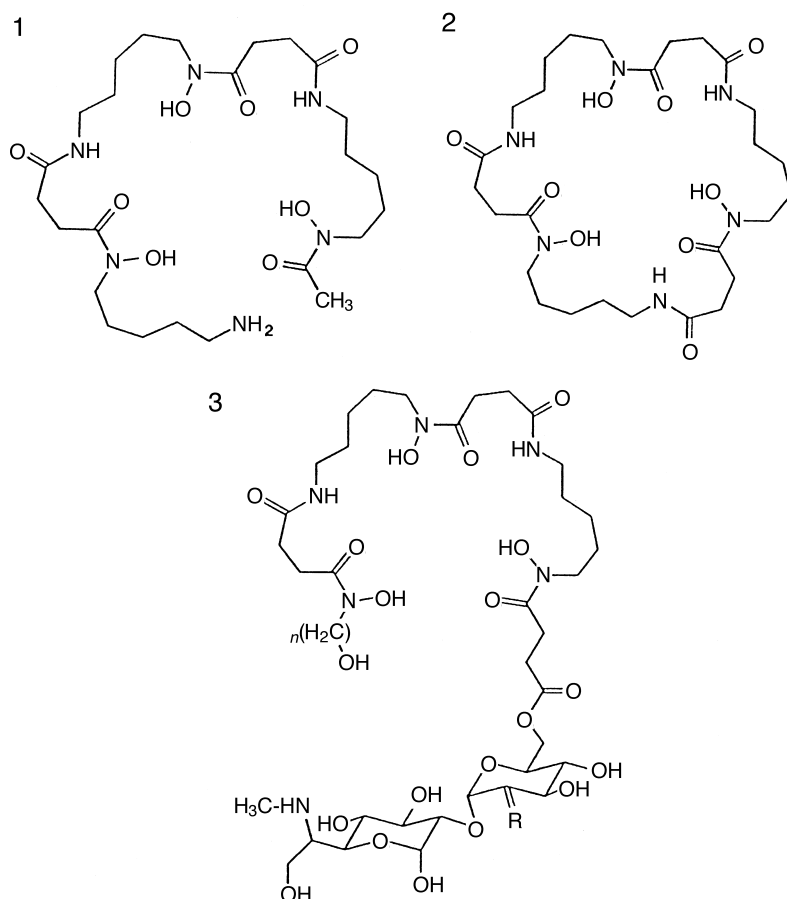


Fig. 10.2. Structure of some desferrioxamines: DFO B (Desferal) (1); DFO E (2); salmycin A (3) (R = NOH, $n = 5$). Salmycin is a natural DFO antagonist produced by *Streptomyces violaceus*.

iron(III), can react in a Fenton reaction, in which $\text{H}_2\text{O}_2 + \text{Fe}^{2+}$ gives $\text{Fe}^{3+} + \text{OH}^- + \text{OH}^\bullet$, the hydroxyl radical being very toxic to macromolecules (see Fig. 10.4) (Halliwell and Gutteridge, 1987). In addition, catechol is susceptible to oxidation and, unlike hydroxamate, is capable of forming uncharged complexes, which are able to undergo intramolecular electron-transfer reactions. The redox state of iron coordinated to catechol is dependent on the external pH and can be repeatedly cycled between iron(II) and iron(III) complexes upon pH variation (Hider, 1984).

Structural characteristics of siderophores influence the stoichiometry and the stability of ferric and ferrous complexes in solution. Therefore, depending on the biological context, the ferric complex of a siderophore may generate secondary effects that can be of physiological importance. This may explain the

prevalence of aerobactin as a virulence factor in clinical infection caused by enterobacterial pathogens, compared with that of enterobactin (Bullen and Griffiths, 1987; de Lorenzo and Martinez, 1988; Lafont *et al.*, 1987), even though enterobactin, due to a higher affinity for iron than aerobactin (K_f for enterobactin = 10^{52} , K_f for aerobactin = 10^{23}), should be more competitive in the presence of serum transferrin ($K_f = 10^{29}$). The presence in serum of anti-enterobactin antibodies, the binding of enterobactin to albumin, the relatively low water solubility of enterobactin and the chemical instability of the enterobactin molecule may impede the effectiveness of enterobactin-mediated iron transport during infection. Chrysobactin and achromobactin both appear to be involved in the pathogenicity of *E. chrysanthemi* strain 3937. Interestingly, the ferric chelates of these two siderophores react differently in the presence of hydrogen peroxide (D. Expert, unpublished result). The case of DFO produced by *E. amylovora* provides another good illustration of how the role of a siderophore can be influenced by its stability.

Role in pathogenicity of desferrioxamine E, the major siderophore of *E. amylovora*

A clear-cut analysis of the role of iron in pathogenicity of *E. amylovora* is not possible without characterizing the high-affinity iron uptake system(s) produced by this bacterium in iron-limited environments. The first data (Vanneste and Expert, 1990), indicating that the production of hydroxamate was correlated with the presence of two low-iron-inducible bacterial outer-membrane proteins in *E. amylovora* strain CFBP 1430, have prompted several groups to further analyse the structure of the siderophore(s) involved. Compounds of the DFO family, of which DFO E appeared to be predominantly produced, were identified in various strains of *E. amylovora* (Feistner *et al.*, 1993; Kachadourian *et al.*, 1996) (see Fig. 10.2). All members of this class of hydroxamate siderophores contain repeating units of 1-amino- ω -N-hydroxy-aminoalkane (pentane or butane), alternated with succinic or acetic acids as acyl functions. Both linear (DFO A₁₋₂, B, C, F, G₁₋₂ and H) and cyclic members (DFO E, D₂, X₁₋₂₋₇ and T₁₋₂) are known. DFO E (nocardamin), the hydroxamate-type siderophore which has the highest complexation constant for Fe(III) ($K_f = 10^{32.5}$), was first isolated in actinomycetes (Yang and Leong, 1982; Müller and Raymond, 1984) and then found in other bacteria, including *Chromobacterium violaceum*, *Pseudomonas stutzeri*, *Hafnia alvei* and *Erwinia herbicola* (Müller and Zähler, 1968; Meyer and Abdallah, 1980; Berner *et al.*, 1988; Reissbrodt *et al.*, 1990). In addition to DFO E, *E. amylovora* releases small amounts of DFO D₂, X₁₋₇ and G₁, which may be considered as minor metabolites (Feistner *et al.*, 1993; Kachadourian *et al.*, 1996).

Among microbial siderophores, DFO are particularly interesting molecules. DFO B (see Fig. 10.2), widely used since 1962 in the treatment of human iron overload and iron poisoning, has attracted much attention as a laboratory tool,

because of the possible involvement in several human diseases of iron in the production of oxyradical mediators of tissue injuries (for a review, see Halliwell, 1989). The mesylate salt of DFO B is available commercially under the trade name Desferal (Ciba-Geigy). DFO B was found to protect against oxidative stress damage in animal tissues. Because of the high stability of the ferrioxamine complex, the iron might be blocked at the ferric oxidation state, which would inhibit the generation of hydroxyl radicals via the Fenton reaction. On the other hand, high concentrations of the free ligand DFO can react *in vitro* directly with $O_2^{\circ-}$ and H_2O_2 or act as a substrate for peroxidase in the presence of H_2O_2 , leading in both cases to the nitroxide radical of DFO E. The latter might be responsible for the toxic secondary effects sometimes observed *in vivo* (Davies *et al.*, 1987; Morehouse *et al.*, 1987). As most plant defence reactions against pathogens involve the generation of harmful reactive oxygen species, such as the superoxide radical anion hydrogen peroxide (Baker and Orlandi, 1995; Low and Merida, 1996), it was of interest to investigate the role of the DFO-mediated iron uptake system of *E. amylovora* during the development of disease. Furthermore, *E. amylovora*, like *E. chrysanthemi*, does not have a specific transport for the ferric complex of citrate, which is one of the major iron sources in plants (Brown, 1978).

The role of DFO-mediated iron uptake in the development of fire blight was unravelled by studying the virulence properties of two transposon-induced mutants of strain CFBP 1430 affected in DFO-mediated iron transport (Dellagi *et al.*, 1998). One mutation disrupts the DFO biosynthesis (*dfoA*-61::MudIIpR13), the other one (*foxR*-17::MudIIpR13) affects the synthesis of the specific ferrioxamine receptor FoxR (Kachadourian *et al.*, 1996). The FoxR receptor is one of the two previously mentioned low-iron-inducible outer-membrane polypeptides, i.e. a 70,000-Da protein, which is required for the passage of ferrioxamines from the environment into the periplasm (Kachadourian *et al.*, 1996) (Fig. 10.3). A *foxR* mutant accumulates DFO in the external medium, which decreases iron availability. In the presence of strong iron chelators, such as ethylenediamine-*N*-*N'*-bis(2-(hydroxyphenyl)acetic acid (EDDHA), both mutants are deeply affected in their growth rates. In contrast, the presence of micromolar traces of iron has little effect on the growth of the DFO biosynthetic mutant, showing that DFO is essentially required when iron is strongly liganded. However, the presence of micromolar traces of iron allows the DFO biosynthetic mutant to grow almost as well as the wild-type strain, presumably because some iron gets into the cell independently of the DFO-mediated iron-uptake system. In the same conditions, the growth rate of the *foxR* mutant is still deeply affected, presumably because all the iron is ligated to DFO and kept outside the cell. Therefore, these two mutants display complementary phenotypes, their difference residing in the absence or accumulation of DFO in the medium.

Several assays have been devised for screening the virulence properties of *E. amylovora* mutants (Vanneste *et al.*, 1990). However, as they involve artificial wounding, early events of the infectious process, such as the epiphytic

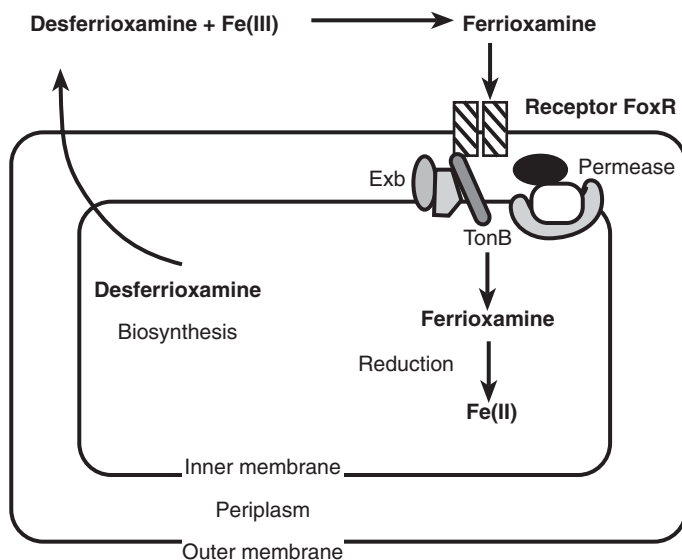


Fig. 10.3. Desferrioxamine-mediated iron acquisition in *Erwinia amylovora*. Details are in the text. Transport proteins, including the TonB machinery and permease (ABC transporter for ferrioxamine), have not yet been characterized in *E. amylovora*.

colonization of the stigmatic surfaces by the pathogen, may escape detection (for a review, see Vanneste, 1995). The ability of *E. amylovora* to directly invade the nectaries (Thomson, 1986, Chapter 2) allowed analysis of the mutants' behaviour after infection of apple flowers, i.e. in conditions closer to the most common naturally occurring situations (Dellagi *et al.*, 1998). Experiments were conducted in the greenhouse on flowering branches during two consecutive springs. Both mutants were shown to be strongly affected in their ability to colonize the flowers: population levels of these mutants were diminished by two orders of magnitude relative to the parental strain, and they were also strongly affected in their ability to initiate necrosis. This indicates that iron is a limiting factor for epiphytic colonization of flower tissues by the pathogen, leading to a lower level of virulence. This is a similar conclusion to that found for *E. chrysanthemi* after studies on the pathogenicity of *E. chrysanthemi* mutants defective in chrysobactin-dependent iron transport. Further evidence that iron is not accessible to the pathogen is provided by the detection of β -galactosidase activity in plant tissues infected with an engineered strain of *E. amylovora* carrying a *foxR-lacZ* gene fusion (Dellagi *et al.*, 1999). In addition, it was found that the failure of the *dfo* mutant to produce symptoms, unlike the *foxR* mutant, is strongly dependent on the inoculum concentration. At a concentration of 10^8 colony-forming units (cfu) ml^{-1} , there are often symptoms. From this and the fact that the response triggered by both mutants was a reaction of 'all or nothing', it was

concluded that the lack of DFO is critical at the onset of infection and can be overcome by increasing the initial inoculum.

Taking into account the property of DFO to interfere with reactive oxygen species, experiments have been conducted to analyse the ability of the *dfo* mutant to provoke electrolyte leakage of leaf tissues in incompatible and compatible situations (Dellagi *et al.*, 1998). Indeed, *E. amylovora* strains carrying a functional *hrp* gene cluster induce electrolyte leakage from plant cells in both compatible and incompatible reactions (Brisset and Paulin, 1992; Baker *et al.*, 1993; Kim and Beer, Chapter 8), as the result of cell damage. Interestingly, the *dfo* mutant appeared to be highly reduced in its ability to induce electrolyte leakage, but the defect was rescued by the addition of exogenous DFO. However, DFO alone failed to induce electrolyte leakage. It has thus been proposed that DFO, by inhibiting the generation of OH° , acts as an agent of protection of bacterial cells against the toxic effects of reactive oxygen species produced through the oxidative burst at the onset of infection. The reduced ability of the *dfo* mutant to induce electrolyte leakage was thus explained by a transient decrease of bacterial population resulting from the oxidative burst (Fig. 10.4). The survival of the *dfo* mutant, grown under laboratory conditions, appeared to be strongly enhanced by the addition of DFO when exposed to lethal doses of H_2O_2 , which supports this hypothesis (Dellagi *et al.*, 1998). However, it cannot be excluded that this siderophore can also generate nitroxide radicals, which contribute to plant cell membrane damage. At a critical inoculum, DFO could therefore enhance the oxidative stress induced by harpin, thus resulting in an overall increase of electrolyte leakage. It is noteworthy that the two mentioned roles of DFO occur at different concentration levels: low levels (1–10 μM range) lead to a protective effect, higher levels, in the presence of peroxidase, are deleterious. As *E. amylovora* cells have the ability to control precisely the synthesis of DFO in intercellular spaces of plant tissues, it is possible that both effects occur during pathogenesis.

***E. amylovora* desferrioxamine-dependent iron uptake: molecular studies**

Desferrioxamine biosynthesis

Although numerous bacterial species produce DFO, the enzymes involved in their biosynthesis, as well as the genes for their cognate receptor, are still unknown. In contrast, probably because of the exploitation of DFO in clinical medicine, a number of chemical syntheses (Bergeron and McManis, 1990; Bergeron *et al.*, 1994) have been reported since Keller-Schierlein and Prelog (1962) described the cyclization of ferrioxamine G_1 into ferrioxamine E. Recently, DFO E was elegantly synthesized from the precursor succinylamino(*N* hydroxy)aminopentane by a one-step cyclic trimerization approach, using iron(III) or gallium(III) as template (Kachadourian *et al.*, 1997). In

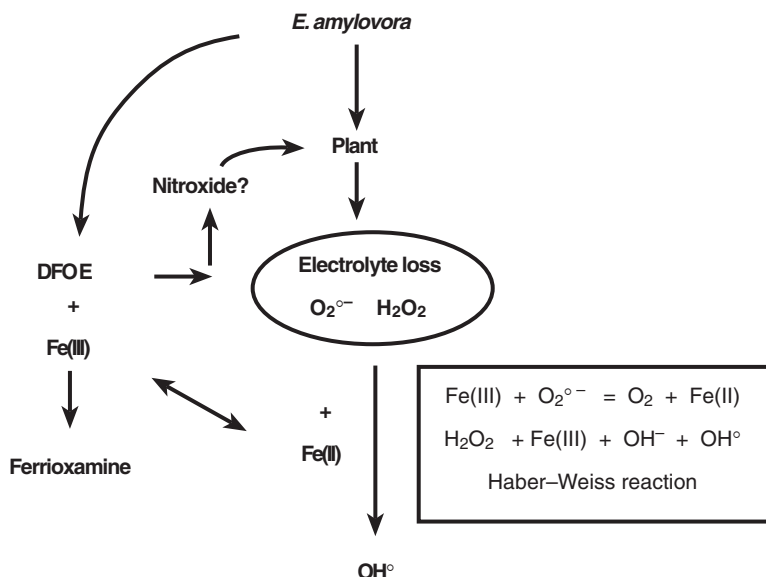


Fig. 10.4. Model showing potential roles of DFO in plant host–*Erwinia amylovora* interactions. Infection by *E. amylovora* of plant tissues causes electrolyte leakage and accumulation of active oxygen species, including the superoxide radical anion and hydrogen peroxide ($O_2^{\cdot-}$ and H_2O_2). They act as antimicrobial agents directly or indirectly through the production of hydroxyl radicals (OH°) generated by the Fenton reaction during the Haber–Weiss cycle catalysed by iron. Iron complexation by DFO interrupts the chain radical reactions, thereby protecting bacterial cells and possibly plant cells from extensive oxidative damage. In addition, depending on the ratios of $Fe(III)$ and $O_2^{\cdot-}$ to DFO, DFO could react with $O_2^{\cdot-}$ to generate the DFO nitroxide radical, which might activate the plant defence mechanism.

E. amylovora, alternative pathways for DFO biosynthesis, using as precursors basic amino acids, such as lysine, ornithine, arginine or diaminopentane, have been proposed. By using a series of potential precursors and 5-hydroxylysine as an inhibitor of biosynthesis, Feistner (1995) provided experimental evidence that decarboxylation of lysine into cadaverine is likely to be the primary step in this pathway (Fig. 10.5). In *Streptomyces pilosus*, a gene encoding a lysine decarboxylase has been identified (Schupp *et al.*, 1988). In *E. amylovora* CFBP 1430, the *dfoA*-61 mutation affecting DFO biosynthesis disrupts a gene that shares identity with the *alcA* gene required for the synthesis of the siderophore alcaligin in *Bordetella* species (Dellagi *et al.*, 1998). This product is a good candidate to catalyse the reaction of *N*-hydroxylation enabling the conversion of cadaverine to 1-amino-5-(*N*-hydroxy)aminopentane (see Fig. 10.5). In addition, several cosmids isolated from a wild-type gene library of *E. amylovora* were shown to restore DFO prototrophy to all *dfo* mutants and to confer the ability to produce

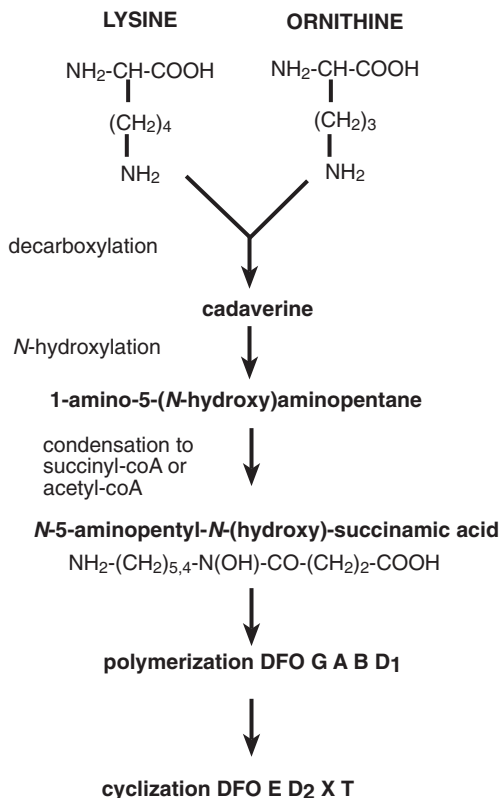


Fig. 10.5. Potential pathway for biosynthesis of desferrioxamines in *Erwinia amylovora*. Details are in the text.

DFO by *Escherichia coli*, suggesting that DFO biosynthetic genes are clustered on the *E. amylovora* chromosome (Dellagi *et al.*, 1998).

Ferrioxamine transport

In Gram-negative bacteria, the specificity of ferrisiderophore uptake has been shown to be at least partially determined by the outer-membrane receptor protein (see Fig. 10.3). The subsequent steps are mediated by less specific ABC-type transporters (for a review, see Tam and Saier, 1993). Transport through the outer membrane has an absolute requirement for the TonB, ExbB and ExbD functions (for a review, see Postle, 1993). The TonB protein is anchored in the cytoplasmic membrane and spans the periplasm to interact directly with outer membrane receptors. Together, these three proteins are thought to form a

complex that energizes the outer membrane by transducing the cytoplasmic membrane-generated proton motive-force energy. The *E. coli* ferrienterobactin and ferrichrome receptors have been extensively studied (Rutz *et al.*, 1992; Killmann *et al.*, 1993). These ligands have been shown to bind the receptor by a relatively short exposed sequence. Deletions in this region transform the ligand-specific high-affinity receptor into a general diffusion pore.

Ferrioxamine receptors have been identified in several bacterial species that do not produce DFO. In *E. coli*, the FhuE protein characterized as the coprogen receptor mediates the transport of ferrioxamines B and E (Sauer *et al.*, 1990). The FoxA receptors of *Pantoea agglomerans* (*E. herbicola*) (Berner and Winkelmann, 1990; Matzanke *et al.*, 1991) and of *Yersinia enterocolitica* (Bäumler and Hantke, 1992) recognize different members of the ferrioxamine family, including ferrioxamines B and E. FoxA of *Y. enterocolitica* shares greater sequence similarity with FhuA, the *E. coli* ferrichrome receptor, than with FhuE, thus validating the concept that other structural constraints, rather than the strict specificity of the ferrisiderophore, have prevailed during evolution in sequence conservation (Bäumler and Hantke, 1992). Interestingly, the FoxR receptor of *E. amylovora* also recognizes ferrioxamines B and E. The cognate *foxR* gene has recently been cloned (Kachadourian *et al.*, 1996) and sequenced (Dellagi *et al.*, 1998). The amino acid sequence of FoxR shares 65% identity and 77% similarity with FoxA of *Y. enterocolitica*, which is the highest score found between two receptors recognizing the same substrate in different bacterial genera. In addition, FoxR and FoxA share higher identity with the *E. chrysanthemi* ferrichrysobactin receptor Fct and FhuA (36% and 35%, respectively) than with any other analysed ferrisiderophore receptors (Sauvage *et al.*, 1996). The FoxR sequence displays all the characteristics defined for TonB-dependent proteins and for porins regarding their transmembrane organization. In topological models for porins, variable sequences form loops, whereas more conserved amphipathic regions form membrane-spanning β strands. A multiple alignment of FoxR, FoxA, Fct and FhuA indicates that the eighth external loop of FhuA known to interact with its ligand is well conserved among these receptors. Thus, it will be interesting to examine the corresponding loops in Fct and FoxA for their role in receptor activity and specificity (Dellagi, 1997).

In iron-depleted cells, the *foxR* gene is transcribed into a monocistronic mRNA of about 2.4 kb, which is in agreement with the presence in the *foxR* promoter region of a potential Fur-binding site (Dellagi, 1997). The Fur protein, identified in many bacteria, acts as a transcriptional repressor, in the presence of ferrous iron, of iron-regulated genes (de Lorenzo *et al.*, 1987).

Potential exploitation of the *E. amylovora* desferrioxamine-dependent iron uptake pathway for fire blight control

Our increasing knowledge of the biochemistry and molecular biology of the iron-scavenging and transporting pathway in *E. amylovora*, as well as its

importance in pathogenicity, allows the development of novel control strategies against fire blight to be considered. For instance, chemical methods based on the design and synthesis of drugs inhibiting DFO biosynthesis or using the ferri-oxamine transport machinery as a specific delivery system constitute attractive approaches (Feistner, 1995; Kachadourian *et al.*, 1996). Interestingly, the reaction of *N*-hydroxylation in the DFO biosynthetic pathway occurs in microorganisms only and the search for specific inhibitors may be fruitful.

The possibility of using high-affinity iron transport pathways as permeation systems (Miller, 1989; Shanzer *et al.*, 1991) has received much attention from the medical world, because the development of methods to facilitate active transport of antibiotics into microbial cells is an important therapeutic goal. Conjugation of antibiotics to siderophore is not a new concept. Natural siderophore-antibiotic combinations have been discovered. Albomycin (Hartmann *et al.*, 1979) and ferrimycin A₁ (Bickel *et al.*, 1960) contain structural components related to desferrichrome and DFO, respectively, and a covalently attached antimicrobial agent that exerts its activity after being delivered into the cell by the iron transport system. Ferrimycins have not found technical applications, because of their chemical instability, but salmycins, which have been characterized only recently (Vértesy *et al.*, 1995) (see Fig. 10.2), would be interesting to investigate at the biological level. The structural complexity of natural siderophores may have impeded progress in the synthesis of such compounds. However, the progress on siderophore chemistry, as well as ferrisiderophore transport, augments the feasibility of this approach.

The finding that desferrioxamine interferes with the oxidative burst elicited by bacteria opens a new field of investigation. The interference of DFO with reactive oxygen species may result in more or less specific defence reactions from the plant, which need to be elucidated. Further understanding of underlying mechanisms may be useful for the development of resistant plants.

Concluding remarks

Studying the role of iron in *E. amylovora* pathogenicity has greatly contributed to the elucidation of the role of siderophores in plant pathogenesis (Expert, 1999). Indeed, as in animal infections, the role of siderophores in the virulence of plant pathogens appears to be more subtle than might be expected and intimately related to the life cycle of the pathogen within its host. Siderophores have now been shown to be implicated in plant disorders as diverse as smut disease, caused by *Ustilago maydis*, crown-gall and necrosis of cherry fruits, induced by *Agrobacterium tumefaciens* and *Pseudomonas syringae*, respectively (for a review, see Expert *et al.*, 1996). The studies on the soft-rot disease elicited by *E. chrysanthemi* have shown that iron is not readily available in plant tissues for invading microorganisms. It was demonstrated that the availability of iron limits substantial growth and movement of bacteria inside the plant (Enard *et al.*, 1988; Masclaux and Expert, 1995). *E. chrysanthemi* mutants blocked in both

chrysobactin- and achromobactin-mediated iron acquisition are deeply affected in their virulence and yet remain able to produce detectable amounts of maceration (C. Enard, 1997, personal communication). Pectin breakdown by *E. chrysanthemi* enzymes and subsequent cell wall deconstruction represent a powerful strategy, allowing bacteria free access to plant nutrients and weakening plant cells. Recent studies have shown the occurrence of a regulatory network coordinating both virulence functions, iron transport and pectinolysis (Masclaux *et al.*, 1996; Expert, 1999). In contrast, *E. amylovora* does not release pectinases, and mutants defective in DFO-mediated iron uptake can fail to produce disease symptoms, indicating that the outcome of infection is dependent on the iron pool of bacterial cells. This fact is important in terms of regulation of iron acquisition, as well as pathogenicity. Furthermore, the interference of DFO with the oxidative burst allows consideration of this siderophore as a full virulence factor, the production of which must be accurately controlled *in planta*. This point illustrates a second facet of the physiological roles of siderophores. It seems that DFO has been harmoniously designed to fulfil the dual function of a powerful iron transporter and a virulence factor. Interestingly, it is possible that in other bacterial species, such as *E. chrysanthemi*, two different siderophores, which are structurally less sophisticated than DFO, fulfil similar complementary functions during pathogenesis.

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Control of Fire Blight



Chemical Control of Fire Blight

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Introduction

Chemicals are applied either to eliminate or to inactivate plant-pathogenic bacteria before these bacteria succeed in penetrating the host tissues. They are also applied to render the plant surfaces unsuitable for the establishment of new infections. In the case of *Erwinia amylovora*, these goals are achieved by destroying the source of inoculum, such as overwintering cankers and alternative hosts, or by protecting potential invasion sites, such as blossoms, stomata, nectarthodes, lenticels or wounds. Therefore, to be effective against *E. amylovora*, bactericides should be applied during three distinct periods of the host life cycle: when the plant is dormant, when it is in bloom and post-bloom.

During the dormant period, in autumn and in spring before bloom or bud break, chemical applications aim to reduce inoculum or to inhibit the multiplication of *E. amylovora*, which overwinters in cankers, thus preventing the development of new blossom infections. Since the risk of phytotoxicity is low during dormancy, it is recommended to use high concentrations of pesticides. This will enhance their activity and persistence.

Chemical sprays during the blooming period aim to protect flowers from infection and prevent a build-up of inoculum for shoot infections. At this stage, the lowest effective concentrations are recommended, because flowers, young leaves and shoots are highly susceptible to phytotoxicity.

Post-bloom sprays during summer are recommended to protect secondary blossoms, common in some pear varieties, from infection when weather conditions are favourable.

After fire blight has been established, chemicals have no significant effect on the progress of the disease since most of the available bactericides have no

systemic action. They do not have the ability to penetrate and move through plant tissues to act against the bacteria. However, some chemicals may completely (e.g. (fosetyl-Al)) or partially (e.g. some antibiotics) penetrate plant tissues. The same is true for some novel compounds, e.g. 1,2,3-benzothiadiazole-7-carbothioic acid-*S*-methyl ester (BTH) (Novartis Crop Protection), sold as BionTM or ActigardTM, harpin, a protein produced by *E. amylovora*, sold as MessengerTM by Eden Bioscience Corp., and prohexadione-Ca, sold as ApogeeTM by BASF Crop, which do not have any bactericidal activity, but which interfere with plant metabolism, either triggering the plant defence mechanism (BionTM, MessengerTM), leading to systemic acquired resistance (SAR), or suppressing shoot growth (ApogeeTM) and thus lowering shoot susceptibility to infection. The application of these compounds should be timed before infection occurs for the plant to respond effectively.

Although fire blight has been known for more than 200 years and most, if not all, developed bactericides have been tested against this disease, no satisfactory and reliable spray programme has been developed that can be recommended for field application. There are many reasons for this, but the main reason could be the nature of the disease and the lack of effective, commercially available, environmentally safe and non-phytotoxic systemic bactericides.

E. amylovora has a wide host range, infecting many plants belonging to the *Rosaceae* family and especially the subfamily of *Pomoideae* (Momol and Aldwinckle, Chapter 4). Many of these plants are wild or grown as ornamentals in areas where cultivated host species are also planted. Under favourable climatic conditions, the primary inoculum builds up quickly on these hosts, giving rise to epidemics that are difficult to control. It is necessary to have an accurate and reliable prediction system in order to time sprays effectively (Billing, Chapter 15). Bactericides need to be applied before the inoculum reaches the receptive plant sites, and need to remain active as long as the inoculum is present. The number of sprays depends on the weather conditions (rain, hail, temperature) and the length of period favourable for initiation of infection (blooming period).

The application of bactericides should be combined with other measures, as part of an integrated programme, where cultural measures, such as proper irrigation, pruning and fertilization, as well as biological agents, may be involved (Steiner, Chapter 17). Proper variety and rootstock selection for plantation in areas with fire blight history should also be taken into consideration (Lespinnasse and Aldwinckle, Chapter 13).

Chemical control of fire blight gave inconsistent results in field experiments, and clear conclusions about the effectiveness and phytotoxicity of a specific compound cannot always be drawn. Some of the reasons explaining the variability of the results are the level of inoculum, the time of application, the weather conditions, the plant species or cultivar, the method of application and the physiological state of the host plant. The inoculum level plays an important role: the higher the inoculum pressure, the less effective a chemical is (Koistra and de Gruyter, 1984; Tsiantos and Psallidas, 1996a). Since, with few exceptions (perhaps streptomycin, which seems to have some curative action), all

bactericides available are preventive and those which are bactericidal do not penetrate the plant tissues, the timing of application is critical for the control of *E. amylovora* as well as other bacterial diseases. The time of application and the physiological state of the plant also affect the host plant response and phytotoxic effect. Young leaves, flowers and fruits have different tolerance and dose response to a chemical. To prevent infection, the bactericides should be present at the infection site before the pathogen. The length of time the chemical stays active is therefore critical and should be taken into consideration when control experiments are scheduled. Weather conditions, especially temperature and humidity, affect the activity of the chemical, especially its phytotoxicity. The method of application is also very important since the chemical should reach all potential sites of infection (upper and lower leaf surface, flowers, etc.).

Chemicals used against fire blight

A large number of chemicals have been tested against fire blight. Van der Zwet and Keil (1979), summarizing the data on chemical control of fire blight on apples and pears published from 1920 to 1979, grouped them into four categories: copper compounds, antibiotics, carbamates and miscellaneous compounds. Most of these results refer to experiments performed in North America (USA and Canada), except for two references from New Zealand and one from Denmark. After the establishment of fire blight in Europe and its spread to different countries, the research for its chemical control boomed and some new compounds, along with the old ones recommended against the disease in North America, were tested for their effectiveness under European environmental conditions. A summary of the data from these experiments is presented in Tables 11.1, 11.2 and 11.3.

Combining the data presented by van der Zwet and Keil (1979) and those of Table 11.1, it is obvious that the two groups of chemicals that have the most important role in controlling fire blight on apples and pears are copper compounds and antibiotics. There are also some novel compounds that could be used against fire blight, overcoming some of the disadvantages of copper and antibiotics; such as flumequin, fosetyl-Al, an oxolinic acid derivative, and also harpin, prohexadione Ca and BTH (1,2,3-benzothiadiazole-7-carbothionic acid-S-methyl ester) (Steiner, Chapter 17). It needs to be noted that, of the vast number of chemicals tested against fire blight, only a few have been registered for control of fire blight in countries with fire blight history (Table 11.4).

Copper compounds

Copper compounds have been established as effective bactericides and have been used against fire blight on apples and pears since 1900 (van der Zwet and Keil,

text continued on p. 216

Table 11.1. Chemical compounds used in experiments to control fire blight on pomaceous plants from 1980 to 1996.

Common name	Commercial name	Dosage	Disease type	Host	Effectiveness (% control)	Country	Year	References
A. Copper compounds Ammoniacal copper sulphate	Copac E	7.5 l ha ⁻¹	Blossom blight*	Pear ^a	100	BE	1982	Mappes <i>et al.</i> (1984)
	Copac E	200–300 ml 100 l ⁻¹	Blossom blight*	Pear ^a	NS	EG	1990	El Nasr <i>et al.</i> (1990)
	Copac E	14 ml 100 l ⁻¹	Shoot blight*	Pear ^b	43	BE	1987	Deckers <i>et al.</i> (1987a)
	Copac E	14 ml 100 l ⁻¹	Blossom blight*	Pear ^a	35–100	BE	1984/85	Deckers <i>et al.</i> (1987a)
	Copac E	6–7.5 l ha ⁻¹	Blossom blight*	Pear ^a	75–96	CY	1989	Dimova-Aziz (1989)
	Copac E	15 ml 100 l ⁻¹	Blossom blight*	Cider apple ^a	NS	GB	1985	Jones and Byrde (1987)
	Copac E	500 ml 100 l ⁻¹	Blossom blight*	Pear ^b	Medium	GR	1993	Tsiantos and Psallidas (1993a)
	Copac E	500 ml 100 l ⁻¹	Fire blight*	Pear ^a	Good	BE	1983	Deckers and Porreye (1984)
	Copac E	150 p.p.m. Cu	Blossom blight*	Cotoneaster ^a	45	NL	1983	Koistra and de Gruyter (1984)
	Copac E	150 p.p.m. Cu	Blossom blight*	Pear ^a	32	NL	1983	Koistra and de Gruyter (1984)
Copper	Copac E	300, 150 p.p.m. Cu	Blossom blight*	Cotoneaster ^b	NS	FR	1981–1983	Paulin and Lachaud (1984)
	Copac E		Fire blight*	Pear ^a	74–89	TR	1991	Momol and Yegen (1993)
	Copac E	750 ml ha ⁻¹	Blossom blight*	Pear ^b	64.6	TR	1993	Demir and Gundogdu (1993)
	Lab002828F	112 p.p.m.	Blossom blight*	<i>Cotoneaster salicifolius</i> ^b	63.3	DE	1982	Zeller <i>et al.</i> (1984)
	Lab002828F	112 p.p.m.	Shoot blight*	Pear rootstock ^b	73.5	DE	1982	Zeller <i>et al.</i> (1984)
	Lab002828F	150 p.p.m.	Shoot blight*	Pear rootstock ^b	81.5	DE	1983	Zeller <i>et al.</i> (1984)
	URA 001400	100 p.p.m.	Shoot blight*	Pear rootstock ^b	54.7	DE	1982	Zeller <i>et al.</i> (1984)

Copper hydroxide	Champion 21	100–500 g 100 l ⁻¹	Blossom blight*	Pear ^b	Good	PL	1990	Sobiczewski and Berczynski (1990)
	Champion 50	400 g 100 l ⁻¹	–	Pear ^c	79	TR	1993	Saygili and Üstün (1996)
	Kocide 101	200 g 100 l ⁻¹	Blossom blight*	<i>Cotoneaster dameri</i> ^b	8	NL	1979	Koistra and Langeslang (1981)
	Kocide 101	200 g 100 l ⁻¹	Blossom blight*	<i>C. salicifolius</i> ^b	93	NL	1980	Koistra and Langeslang (1981)
	Kocide 77WP	0.48 kg 100 l ⁻¹	Canker [†]	Apple ^a	Good	US	1984	Burr and Norelli (1984)
	Kocide 101	1 p.p.m. Cu	Blossom blight*	Pear ^b	Low	FR	1985	Paulin <i>et al.</i> (1987)
	Kocide 101	200 g 100 l ⁻¹	Blossom blight*	Pear ^b	Medium	GR	1993	Tsiantos and Psallidas (1993a,b)
	Kocide 101	200 g 100 l ⁻¹	Blossom blight [†]	Pear ^b	Low	GR	1993	Tsiantos and Psallidas (1993a,b)
	Kocide 101	120 mg 100 l ⁻¹	Blossom blight* [†]	Pear ^b	Low	GR	1993	Tsiantos and Psallidas (1993a,b)
	Kocide 101 (50%)	1000 p.p.m. Cu	Blossom blight*	Cotoneaster ^b	NS	FR	1983	Paulin and Lachaud (1984)
Copper oxychloride	Copper (Bayer)	100 g 100 l ⁻¹	Blossom blight*	<i>C. dameri</i> ^b	93	NL	1979	Koistra and Langeslang (1981)
	Copper (Bayer)	200 g 100 l ⁻¹	Shoot blight*	Quince ^b	NS	NL	1980	Koistra and Langeslang (1981)
	Copper (Bayer)	200 g 100 l ⁻¹	Shoot blight*	<i>C. dameri</i> ^b	NS	NL	1980	Koistra and Langeslang (1981)
	Copper (Bayer)	200 g 100 l ⁻¹	Blossom blight*	<i>C. dameri</i> ^b	96	NL	1979	Koistra and Langeslang (1981)
	Copper (Bayer)	200 g 100 l ⁻¹	Blossom blight*	<i>C. dameri</i> ^b	77	NL	1980	Koistra and Langeslang (1981)
	Copper (Bayer)	200 g 100 l ⁻¹	Blossom blight*	<i>C. salicifolius floccosus</i> ^b	100	NL	1980	Koistra and Langeslang (1981)
	Not specified	1000 p.p.m. Cu	Fire blight*	Cotoneaster ^a	61	NL	1989	Coster and Waalkens (1989)

continued

Table 11.1. *continued*

Common name	Commercial name	Dosage	Disease type	Host	Effectiveness (% control)	Country	Year	References
Copper oxychloride sulphate + spray oil Copper oxyquinolate	Not specified	200 g a.i. 100 l ⁻¹	Fire blight*	Pear ^b	Medium	TR	1993	Momol and Yegen (1993)
	Not specified	2500 p.p.m.	Blossom blight*	Pear ^a	NS	EG	1990	El Nasr <i>et al.</i> (1990)
	Cupravit OB21	500 g 100 l ⁻¹	Blossom blight*	Cotoneaster ^b	66	DE	1984	Zeller <i>et al.</i> (1984)
	Cupravit 50	120 g 100 l ⁻¹	Blossom blight*	Apple ^b	74.5	CY	1989	Dimova-Aziz (1989)
	Cupravit 50	500 p.p.m. Cu	Blossom blight*	Cotoneaster ^b	NS	FR	1981	Paulin and Lachaud (1984)
	Mavi bekir	400 g 100 l ⁻¹	Blossom blight*	Pear ^c	NS	TR	1996	Saygili and Üstün (1996)
	Miezidian 50	100–500 g 100 l ⁻¹		Pear ^a	55	PL	1990	Sobiczewski and Berczynski (1990)
	Phytocoper	1000 p.p.m.	Blossom blight*	Cotoneaster ^b	Good	DE	1984	Zeller <i>et al.</i> (1984)
	Virifix 50	200 g 100 l ⁻¹	Blossom blight**†	Pear ^b	NS	GR	1993	Tsiantos and Psallidas (1993a,b)
	Bayer 50%	1000 p.p.m. Cu	Blossom blight*	Cotoneaster ^a	83	NL	1983	Koistra and de Gruyter (1984)
Copper sulphate	Bayer 50%	1000 p.p.m. Cu	Blossom blight*	Cotoneaster ^b	87	NL	1980	Koistra and de Gruyter (1984)
	COCs	320 g 100 l ⁻¹	Canker†	Apple ^a	Good	US	1984	Burr and Norelli (1984)
	Copper oxyquinolate	200 g 100 l ⁻¹	Blossom blight*	Cotoneaster ^b	0–3	NL	1980	Koistra and Langeslang (1981)
Copper sulphate + lime	Copper	400 g 100 l ⁻¹	Blossom blight*	Pear ^c	9–34	TR	1996	Saygili and Üstün (1996)
	Copper	2500 p.p.m.		Pear ^a	NS	EG	1990	El Nasr <i>et al.</i> (1990)
Copper sulphate + lime	Bordeaux mixture	500 g 100 l ⁻¹	Blossom blight*	Pear ^b	Medium	GR	1993	Tsiantos and Psallidas (1993a,b)
	Bordeaux mixture 20%	250 g 100 l ⁻¹	Blossom blight**†	Pear ^b	Low		1993	Tsiantos and Psallidas (1993a,b)
	Bordeaux mixture 20%	1 mg ml ⁻¹ Cu	Blossom blight*	Pear ^b	Medium	FR	1985	Paulin <i>et al.</i> (1985)

Bordeaux mixture + spray oil	0.94–0.94–100	Canker [†]	Apple ^a	Good	US	1984	Burr and Norelli (1984)
Bordeaux spray 20%	500 p.p.m. Cu	Blossom blight*	Cotoneaster ^b	Good	FR	1981–1983	Paulin and Lachaud (1984)
Bordeaux mixture 16%	1000 p.p.m. Cu	Blossom blight*	Cotoneaster ^b	Good	FR	1983	Paulin and Lachaud (1984)
Bordeaux mixture	100 g a.i. 100 l ⁻¹	Blossom blight*	Pear ^b	Medium	FR	1991	Brisset <i>et al.</i> (1991)
Bordeaux mixture	100 g a.i. 100 l ⁻¹	Blossom blight*	Apple ^b	Good	FR	1991	Brisset <i>et al.</i> (1991)
Copper mixtures							
Copper oxychloride + maneb	1500 + 800 a.i. l ⁻¹	Fire blight	Pear ^a	82.47	TR	1991	Momol <i>et al.</i> (1991)
Copper oxychloride Herkul + maneb	400 g 100 l ⁻¹		Pear ^c	100	TR	1996	Saygili and Üstün (1996)
Copper oxychloride Dacobre + chlorothalonil	400 g 100 l ⁻¹		Pear ^c	25–75	TR	1996	Saygili and Üstün (1996)
3 Copper salts + mancozeb	400 g 100 l ⁻¹		Pear ^c	98	TR	1996	Saygili and Üstün (1996)
3 Copper salts (21.5 + 20%)							
3 Copper salts + maneb	840 p.p.m. a.i.	Blossom blight [†]	Cotoneaster ^b	81	NL	1983	Koistra and de Gruyter (1984)
3 Copper salts + mancozeb	1000 p.p.m. Cu	Blossom blight*	Cotoneaster ^b	Good	FR	1981	Paulin and Lachaud (1984)
3 Copper salts + maneb	860 p.p.m. Cu	Blossom blight*	Cotoneaster ^a	95	NL	1983	Koistra and de Gruyter (1984)
Copper sulphate + foliar fertilizers	31 p.p.m. Cu	Blossom blight [†] and shoot blight	Apple ^b	Medium	US	1993	Clarke <i>et al.</i> (1993)
(21.5 + 20%)							

continued

Table 11.1. continued

Common name	Commercial name	Dosage	Disease type	Host	Effectiveness (% control)	Country	Year	References
Copper sulphate 18% + oligoelements	Cuivrol®	50 g a.i. 100 l ⁻¹	Blossom blight	Apple ^b	Good	FR	1993	Larue and Ardigier (1993)
Copper oxychloride + kasugamycin	Kasumin-Bordeaux (5 + 45%)		Blossom blight	Pear ^b	Good ^d	US	1984	Bonn (1984)
Cuprous oxide	Copper (Sandoz)	200 g 100 l ⁻¹	Blossom blight*	<i>C. dimeri</i> ^b	48	NL	1980	Koistra and Langeslang (1981)
	Copper (Sandoz)	200 g 100 l ⁻¹	Blossom blight*	<i>C. dimeri</i> ^b	96	NL	1979	Koistra and Langeslang (1981)
	Copper (Sandoz)	200 g 100 l ⁻¹	Blossom blight*	<i>C. salicifolius flocosus</i> ^b	94	NL	1980	Koistra and Langeslang (1981)
B. Antibiotics								
Kasugamycin	Kasumin	150, 50 p.p.m.	Blossom blight*	Apple ^b	Good	US	1986/87	Aldwinckle and Norelli (1990)
	Kasumin	2000 p.p.m.	Blossom blight*	Pear ^b	Medium	FR	1985	Paulin <i>et al.</i> (1987)
	Kasumin 2%	5000 p.p.m.		Pear ^c	95–100	TR	1993	Saygili and Üstün (1996)
	Kasumin 25%	300 p.p.m.	Blossom blight*	Cotoneaster ^a	88	NL	1983	Koistra and de Gruyter (1984)
	Kasumin 30 g l ⁻¹	600 p.p.m.	Blossom blight [†]	<i>C. dimeri</i> ^b	90, 69	NL	1983	Koistra and de Gruyter (1984)
	Kasumin 25%	150 p.p.m.	Blossom blight [†]	Cotoneaster ^b	87	NL	1983	Koistra and de Gruyter (1984)
	Kasumin 80%	40 p.p.m.	Shoot blight*	Cotoneaster ^b	NS	FR	1981–1983	Paulin and Lachaud (1984)
	Kasumin 2%	4000 p.p.m.	Fire blight*	Pear ^b	Medium	GR	1996	Tsiantos and Psallidas (1996b)
	Kasumin 25% WP	300 p.p.m.	Fire blight**	Cotoneaster ^{a,b}	Very good	NL	1989	Coster and Waalkens (1989)

Oxytetracycline	Mycoshield	200 p.p.m.	Blossom blight*	Apple ^b	Good	US	1994	McManus and Jones (1994)
Polyoxin	Lacon 2%	60 p.p.m.	Blossom blight*	Cotoneaster ^b	NS	FR	1981–1983	Paulin and Lachaud (1984)
Streptomycin	Streptomycin	200 p.p.m.	Blossom blight*	<i>C. salicifolius</i> ^b	82.8	DE	1979	Egli and Zeller (1981)
	Streptomycin	106 p.p.m.	Fire blight*	Cotoneaster ^b	87	NL	1989	Coster and Waalkens (1989)
	Streptomycin	100 p.p.m.	Fire blight†	Cotoneaster ^b	48	NL	1989	Coster and Waalkens (1989)
	Streptomycin	200 p.p.m.	Cell suspension		Good	I	1989	Scottichini and Rossi (1991)
	Streptomycin	100 p.p.m.	Blossom blight*	Pear ^b	78.69	TR	1993	Demir and Gundogdu (1993)
	Agrept 20%	100–200 p.p.m.	Blossom blight*†	Pear ^{a,b}	Good	GR	1993	Tsiantos and Psallidas (1993a,b)
	Agrept	200 p.p.m.	Shoot blight	Pear ^a	Good	GR	1993	Tsiantos and Psallidas (1993a,b)
	Agrimycin 17	100 p.p.m.	Shoot blight*	Pear ^c	Good	PL	1990	Sobiczewski and Berczynski (1990)
	Agri-cycin 17	100 p.p.m.	Shoot blight*	Pear rootstock ^b	83	DE	1982	Zeller <i>et al.</i> (1984)
	Agri-cycin 17	100 p.p.m.	Blossom and shoot blight*	Pear ^b	Good	CA	1984	Bonn (1984)
	Agrimycin 17	100 p.p.m.	Blossom blight*	Apple ^b	Good	US	1994	McManus and Jones (1994)
	Agri-cycin 17	100 p.p.m.	Blossom blight*	Cider apple ^a	90	GB	1985	Jones and Byrde (1987)
	Agri-strep 21.2 WP	100, 50 p.p.m.	Blossom blight*	Apple ^a	100, 98	US	1984	Burr and Norelli (1984)
	Agri-strep 21.2 WP	100 p.p.m.	Blossom blight*	Apple ^b	Good	US	1987	Aldwinckle and Norelli (1990)
	Plantomycin 17	100 p.p.m.	Blossom blight*	Pear ^b	Good	FR	1985	Paulin <i>et al.</i> (1987)
	Plantomycin 17	300, 600 p.p.m.	Blossom blight*	Cotoneaster ^b	98, 96	NL	1979	Koistra and Langeslang (1981)

continued

Table 11.1. *continued*

Common name	Commercial name	Dosage	Disease type	Host	Effectiveness (% control)	Country	Year	References
	Plantomycin 17	100 p.p.m.	Fire blight*	Cotoneaster ^b	67, 100	NL	1980	Koistra and Langeslang (1981)
	Plantomycin 17	100–200 p.p.m.	Blossom blight*	Cotoneaster ^b	NS	FR	1981–1983	Paulin and Lachaud (1984)
	Plantomycin 17	600 p.p.m.	Shoot blight**	<i>C. salicifolius</i> ^b	42, NS	NL	1980	Koistra and Langeslang (1981)
	Plantomycin 17	600 p.p.m.	Shoot blight**	Quince ^b	87, NS	NL	1980	Koistra and Langeslang (1981)
	Plantomycin 17	600 p.p.m.	Blossom blight**	<i>C. salicifolius</i> ^b	99, 92	NL	1980	Koistra and Langeslang (1981)
	Plantomycin 17	600 p.p.m.	Blossom blight**	<i>C. damer</i> ^b	83, 41	NL	1980	Koistra and Langeslang (1981)
	Plantomycin 17	600 p.p.m.	Blossom blight**	Pear ^b	58, 62	NL	1980	Koistra and Langeslang (1981)
	Plantomycin 17	100 p.p.m.	Blossom blight*	Apple ^a	73	FR	1990	Larue and Ardigier (1993)
	Plantomycin 17	100 p.p.m.	Blossom blight*	Pear ^a	63–91	NL	1983	Koistra and de Gruyter (1984)
	Plantomycin 17	100 p.p.m.	Blossom blight*	Cotoneaster ^b	87	NL	1983	Koistra and de Gruyter (1984)
	Plantomycin 17	100 p.p.m.	Blossom blight**	<i>C. damer</i> ^b	79, 23	NL	1983	Koistra and de Gruyter (1984)
	Plantomycin 17	100 p.p.m.	Blossom blight*	Pear ^b , apple ^b	Good	FR	1991	Brisset <i>et al.</i> (1991)
	Streptomycin sulphate	100 p.p.m.	Blossom blight*	<i>C. salicifolius</i> ^b	49.6	DE	1983	Zeller <i>et al.</i> (1984)
	Streptomycin sulphate	100 p.p.m.	Shoot blight*	Pear ^b	75.5	DE	1983	Zeller <i>et al.</i> (1984)
	Streptomycin sulphate	100 p.p.m.	Shoot blight*	Pear ^b	62	BE	1984	Deckers <i>et al.</i> (1987a)

Streptomycin sulphate	100 p.p.m.	Blossom blight*	Pear ^a	2–100	BE	1984/85	Deckers <i>et al.</i> (1987a)
Streptomycin sulphate	100 p.p.m.		Pear ^c	95–100	TR	1996	
Streptomycin sulphate	100 p.p.m.	Fire blight*	Cotoneaster ^b	Medium	BE	1983	Deckers and Porreye (1984)
MBR 10995, 35	600 p.p.m.	Blossom blight*	Apple ^b	94	US	1983	Burr and Norelli (1984)
Fructil TM	350 p.p.m.	Shoot blight*	Pear ^b	70	BE	1987	Deckers <i>et al.</i> (1987a)
Fructil TM	225, 300 p.p.m.	Blossom blight*	Pear ^a	63–100	BE	1984	Deckers <i>et al.</i> (1987a)
MBR 10995 80W	300 g a.i. ha ⁻¹	Fire blight*	Pear ^a	Good	BE	1983	Deckers and Porreye (1984)
MBR 10995 50%	125, 250 p.p.m.	Blossom blight*	Cotoneaster ^b	80, 99	NL	1983	Koistra and de Gruyter (1984)
MBR 10995	600 p.p.m.	Blossom and shoot blight*	Pear ^b	Good	CA	1984	Bonn (1984)
Firestop TM	300 p.p.m.	Blossom blight	Pear ^b	Good	FR	1987	Paulin <i>et al.</i> (1987)
MBR 10995 20%	150 p.p.m.	Blossom blight*	Cotoneaster ^b	NS	FR	1983	Paulin and Lachaud (1984)
Fructil TM 80%	200, 300 g a.i. ha ⁻¹	Fire blight	Pear ^a	Good	BE	1986–1988	Deckers <i>et al.</i> (1990)
Firestop TM	300 p.p.m.	Blossom and shoot blight*	Pear ^b	Good	FR	1991	Brisset <i>et al.</i> (1991)
Firestop TM	300 p.p.m.	Blossom and shoot blight*	Apple ^b	Medium	FR	1991	Brisset <i>et al.</i> (1991)
Firestop TM 20%	300 g a.i. ha ⁻¹	Fire blight*	Pear ^a	Good	BE	1986–1988	Deckers <i>et al.</i> (1990)
Firestop TM 20%	3000 p.p.m.	Blossom blight*	Pear ^b	Good	FR	1990	Brisset <i>et al.</i> (1990)
Firestop TM	300 p.p.m.	Blossom blight*	Apple ^b	Medium	FR	1990	Brisset <i>et al.</i> (1990)
Firestop TM 15%	300 p.p.m.	Shoot blight*	Pear ^a	Good	GR	1993	Tsiantos and Psallidas (1993a)

*continued***C. Other compounds****Flumequin**

Table 11.1. continued

Common name	Commercial name	Dosage	Disease type	Host	Effectiveness (% control)	Country	Year	References
Fosetyl-aluminium	Firestop™ 15%	300 p.p.m.	Blossom blight*†	Pear ^{a,b}	Good	GR	1993	Tsiantos and Psallidas (1993a,b, 1996b)
	Firestop™	300 p.p.m.	Blossom blight*	Apple ^b	Good	FR	1988	Larue and Ardigier (1993)
	BASF 0028	60, 90, 120 p.p.m.	Blossom blight*	Apple ^b	72, 54, 89	US	1980	Burr and Norelli (1984)
	30g/LF							
	MBR 25930, 25	600, 100 p.p.m.	Blossom blight*	Apple ^b	100, 100	US	1983	Burr and Norelli (1984)
	Aliette™ 80%	3–4 g a.i. 100 l ⁻¹	Blossom blight*	Pear, apple ^b	Inconsistent	FR	1986–1988	Paulin <i>et al.</i> (1985)
	Aliette™ 80%	60–120 g a.i. 100 l ⁻¹	Blossom blight*	Apple ^b	NS	US	1991/92	Clarke <i>et al.</i> (1993)
	Aliette™ 80%	60–120 g a.i. 100 l ⁻¹	Blossom blight†	Apple ^b	NS	US	1989–1991	Clarke <i>et al.</i> (1993)
	Aliette™ 80%	300 ml 100 l ⁻¹	Blossom blight*†	Pear ^b	Low	GR	1993	Tsiantos and Psallidas (1993a)
	Aliette™ 80%	300 ml 100 l ⁻¹	Blossom blight*†	Pear ^a	Medium	GR	1993	Tsiantos and Psallidas (1993a)
	Aliette™ 80%	300 ml 100 l ⁻¹	Shoot blight*	Pear ^a	Medium	GR	1993	Tsiantos and Psallidas (1993a)
	Aliette™ 80%	300 g a.i. 100 l ⁻¹	Blossom blight*	Pear ^a	Good	GR	1993	Tsiantos and Psallidas (1993b)
	Aliette™ 80%	300 g a.i. 100 l ⁻¹	Blossom blight*†	Pear ^{a,b}	NS	GR	1991	Tsiantos and Psallidas (1993b, 1996b)
Aliette™	Aliette™ 80%	300 g a.i. 100 l ⁻¹	Blossom blight*	Pear ^b , apple ^b	High	FR	1993	Larue and Gaulliard (1993)
	Aliette™	2240–4480 g 100 l ⁻¹	Blossom blight*	Apple ^b	NS	US	1991/92	Norelli and Aldwinckle (1993)
	Aliette™	320 g a.i. 100 l ⁻¹	Blossom blight*	Pear ^b	73.86	TR	1993	Demir and Gundogdu (1993)
	Aliette™	100–400 g a.i. 100 l ⁻¹		Pear ^c	NS	TR	1996	Saygili and Üstün (1996)

7-Chloro-1-ethyl-6-fluoro-1,4-dihydro-4-exo-3-quinoline carboxylic acid	CGA 78039 50W	300 p.p.m.	Blossom blight*	Apple ^b	98	US	1983	Burr and Norelli (1984)
	CGA 78039 50%	500 g a.i. ha ⁻¹	Fire blight*	Pear ^a	Low	BE	1982	Deckers and Porreye (1984)
	CGA 78039 50%	750 p.p.m.	Blossom blight*	Pear ^a	81	NL	1983	Koistra and de Gruyter (1984)
	CGA 78039	300 p.p.m.	Blossom and shoot blight	Pear ^a	Good	CA	1984	Bonn (1984)
	CGA 78039 50%	200–800 p.p.m.	Blossom blight*	Cotoneaster ^b	Good	DE	1979	Egli and Zeller (1981)
	CGA 78039 50%	2000 p.p.m.	Blossom blight**	<i>C. dameri</i> ^b	79, 22	NL	1980	Koistra and Langeslang (1981)
	CGA 78039 50%	2000 p.p.m.	Blossom blight**	Cotoneaster ^b	100, 95	NL	1980	Koistra and Langeslang (1981)
	CGA 78039 50%	2000 p.p.m.	Shoot blight**	Quince ^a	51–88 NS	NL	1980	Koistra and Langeslang (1981)
	CGA 78039 50%	2000 p.p.m.	Shoot blight*	Cotoneaster ^b	29 NS	NL	1980	Koistra and Langeslang (1981)
	CGA 78039 25%	1000–1000 p.p.m.	Blossom blight*	Cotoneaster ^b	55–100	NL	1979	Koistra and Langeslang (1981)
	CGA 78039 50%	200, 400 p.p.m. a.i.	Blossom blight*	Cotoneaster ^b	NS	FR	1981, 1982	Paulin and Lachaud (1984)
	CGA 78039	500 p.p.m.	Blossom blight*	Perry pear, cider apple ^a	NS	GB	1981, 1983	Gwynne (1984)
	CGD 93600 B	750, 500 p.p.m.	Shoot blight*	Cotoneaster ^b	78.4–75.5	DE	1982	Zeller <i>et al.</i> (1984)
	CGD 93600 B	750, 500 p.p.m.	Blossom blight*	Cotoneaster ^b	95.8–93	DE	1982	Zeller <i>et al.</i> (1984)
	CGD 93600 B	750, 500 p.p.m.	Blossom blight†	Cotoneaster ^b	92.5–93	DE	1982	Zeller <i>et al.</i> (1984)
	CGD 93600 B	750, 500 p.p.m.	Shoot blight†	Pear rootstock ^b	71.6–83	DE	1982	Zeller <i>et al.</i> (1984)
	CGD 93600 B	750–500 p.p.m.	Shoot blight*	Pear rootstock ^b	58–73	DE	1982	Zeller <i>et al.</i> (1984)
	CGD 93600 B	750–500 p.p.m.	Shoot blight*	Pear ^b	63.3	DE	1983	Zeller <i>et al.</i> (1984)
	FSB 8332	50 g a.i. ha ⁻¹	Fire blight*	Pear ^a	Good	BE	1983	Deckers and Porreye (1984)

continued

Table 11.1. *continued*

Common name	Commercial name	Dosage	Disease type	Host	Effectiveness (% control)	Country	Year	References
Oxolinic acid	S-0208	500, 1000 p.p.m.	Blossom blight*	Cotoneaster ^b	NS	FR	1982	Paulin and Lachaud (1984)
	S-0208 (20% WP)	300 p.p.m.	Blossom blight*	Cider apple ^a	99	GB	1987	Jones and Byrde (1987)
	S-0208 (20% WP)	300 g a.i. ha ⁻¹	Blossom blight*	Pear ^a	Good	BE	1986–1988	Briset <i>et al.</i> (1990)
	S-0208 (20% WP)	1500 p.p.m.	Blossom blight*	Apple ^a , Pear ^a	93, 98	CY	1987	Dimova-Aziz (1989)
	S-0208 (20% WP)	300 p.p.m.	Blossom blight*	Pear ^c	Good	PL	1986/87	Sobiczewski and Berczynski (1990)
	S-0208 (20% WP)	300–400 p.p.m.	Fire blight*	Pear ^a	76.9–96.4	EG	1988	El Nasr <i>et al.</i> (1990)
	S-0208	1500 p.p.m.	Blossom blight*†	Pear ^{a,b}	Good	GR	1991	Tsiantos and Psallidas (1993a,b, 1996b)
	S-0208 (20% WP)	1500 p.p.m.	Shoot blight†	Pear ^a	Good	GR	1993	Tsiantos and Psallidas (1993a)
D. Disinfectants								
Acetic acid	Acetic acid	1 M	Apple fruits	Dipping	High	CA	1988	Sholberg <i>et al.</i> (1988)
Acetic acid	Acetic acid	1 M	Apple fruits	Dipping	High	US	1989	Roberts and Reymond (1989)
Benzalkonium chloride	BC	1400 mg ml ⁻¹	Apple fruits	Dipping	High	US	1988	Janiewicz and van der Zwet (1988)
Benzalkonium chloride		2000 p.p.m. + 2500 p.p.m. Orthox77	Apple fruits	Dipping	High	US	1989	Roberts and Reymond (1989)
Citrate buffer	Citrate buffer pH 2.5	0.1 M + 500 p.p.m. DBSA	Apple fruits	Dipping	High	US	1989	Roberts and Reymond (1989)
Ethanol	Ethanol	70	Cutting shears	Dipping (2 min) or spraying	High	US	1987	Beer and Rundle (1987)
Ethanol	Ethanol	70	Cutting shears	Dipping (30 min)	100	DE	1996	Heuberger and Poulos (1953)

Isopropanol	Propionic acid			Hands	Washing	85	DE	1996	Heuberger and Poulos (1953)
	Quaternary ammonium			Boots	Spraying (1 min)	100	DE	1996	Heuberger and Poulos (1953)
	Sodium hypochloride		70	Scalpels	Dipping	High	US	1987	Beer and Rundle (1987)
				Apple fruits	Dipping	High	CA	1985	Sholberg <i>et al.</i> (1988)
			1 M	Cell suspension	Mixing	High	DE	1985	Kleinhempel <i>et al.</i> (1987)
				Cutting shears	Spraying	Medium	DE	1980	Kleinhempel <i>et al.</i> (1987)
			0.025% + 500 p.p.m.	Apple fruits	Dipping	High	US	1989	Roberts and Raymond (1989)
			Orthox77	Cell suspension	Mixing	Good	DE	1987	Kleinhempel <i>et al.</i> (1987)
			5%	Cutting shears	Spraying	Good	DE	1987	Kleinhempel <i>et al.</i> (1987)
			50%	Cutting shears	Dipping (20 min)	100	DE	1996	Hasler <i>et al.</i> (1996)
			3%	Cutting knives	Dipping	Good	US	1987	Beer and Rundle (1987)
			0.05%	Cutting knives	Dipping	Good	US	1988	Janisiewicz and van der Zwet (1988)
			0.05% + 0.25%	Apple fruits	Dipping	Good	US	1988	Janisiewicz and van der Zwet (1988)
			Ortho-x77	Cutting knives	Dipping	100	DE	1996	Heuberger and Poulos (1953)
			4%	Cutting knives	Dipping	100	DE	1996	Heuberger and Poulos (1953)
				Boots	Immersion (5 min)	100	DE	1996	Heuberger and Poulos (1953)
			4%	Boots	Immersion (5 min)	100	DE	1996	Heuberger and Poulos (1953)
				Hands	Washing (50 s)	100	DE	1996	Heuberger and Poulos (1953)
				Hands	Washing (50 s)	100	DE	1996	Heuberger and Poulos (1953)
				Hands	Washing (50 s)	100	DE	1996	Heuberger and Poulos (1953)

* Protective sprays. † Curative sprays. ^a Natural infection. ^b Artificial inoculation. ^c Pear fruit slices test. ^d Highly phytotoxic. NS, No significant control. Country codes according to ISO 3166:1988.

Table 11.2. Plant extracts tested against *Erwinia amylovora* *in vitro* and/or *in vivo*.

Plant species	Activity		References
	<i>In vitro</i>	<i>In vivo</i>	
<i>Ailanthus altissima</i> (tree of heaven)	+	NT	1
<i>Alchemilla vulgaris</i>	+	+	3
<i>Allium sativum</i> (garlic)	+	(+)	1
<i>Allium sativum</i> (garlic)	+	NT	2
<i>Berberis lampergiana</i> (barberry)	+	NT	1
<i>Berberis vulgaris</i> (barberry)	+	+	1
<i>Castanea sativa</i> (European chestnut)	+	NT	1
<i>Citrus bergamia</i> (bergamot)	—	NT	2
<i>Cupressus sempervirens</i> (cypress)	—	NT	2
<i>Fallopia convolvulus</i> (climbing buckwheat)	+	NT	1
<i>Hedera helix</i> (ivy)	+	+	3, 4
<i>Juglans nigra</i> (black walnut)	+	+	1
<i>Juniperus communis</i> (common juniper)	—	NT	2, 5
<i>Mahonia aquifolium</i> (Oregon grape)	+	+	1
<i>Matricaria chamomilla</i> (chamomile)	(+)	NT	2
<i>Mentha × piperita</i> (peppermint)	—	NT	2
<i>Ocimum basilicum</i> (basil)	—	NT	2, 5
<i>Origanum vulgare</i> (oregano)	+	NT	2
<i>Pelargonium odoratissimum</i>	+	NT	1
<i>Pimpinella saxifraga</i> (common burnet saxifrage)	+	NT	1
<i>Pinus sylvestris</i> (Scots pine)	—	NT	2, 5
<i>Polygonum capitatum</i> (polygonum)	+	NT	1
<i>Populus tremula</i> (trembling poplar)	+	NT	1
<i>Potentilla anserina</i> (potentilla)	+	NT	1
<i>Quercus petraea</i> (sessile oak)	+	NT	1
<i>Quercus robur</i> (English oak)	+	NT	1
<i>Reynoutria sachalinensis</i>	+	+	3
<i>Rheum rabowbarum</i>	+	NT	1
<i>Rhus typhina</i>	+	+	1
<i>Rosa canina</i> (dog rose)	—	NT	2, 5
<i>Rosmarinus officinalis</i> (rosemary)	—	NT	2, 5
<i>Ruta graveolens</i> (garden rue)	+	NT	1
<i>Sabucus nigra</i> (elder)	+	NT	1
<i>Salvia officinalis</i> (sage)	+	NT	1
<i>Salvia sclarea</i> (sclarea sage)	—	NT	1, 2
<i>Satureja hortensis</i> (savory)	+	NT	2
<i>Sedum groenlandicum</i>	+	NT	1
<i>Senecio spiculosus</i>	+	NT	1
<i>Thymus vulgaris</i> (white thyme)	(+ ^b)	NT	1, 2
<i>Tilia tomentosa</i> (silver linden)	(+)	NT	2
<i>Viscum album</i>	+	+	3, 4

*, Induced resistant response; +, Good antibacterial activity; (+), moderate antibacterial activity; (+^b), slight antibacterial activity (bacteriostatic); —, no antibacterial activity; NT, not tested.

References: 1. Mosch *et al.*, 1989; 2. Scortichini and Rossi, 1991; 3. Mosch *et al.*, 1993; 4. Mosch *et al.*, 1996; 5. Scortichini and Rossi, 1989.

Table 11.3. Chemical compounds tested against *Erwinia amylovora* without significant effect on the bacterium and/or the disease.

Common name	Commercial name	References
A. Disinfectants		
Alkyldimethylbenzylammonium chloride	Physan 20%	1
	Dimanin A	2
	Dimanin 33.3%	1
Alkyldimethylbenzylammonium chloride + alkyldimethylethylbenzylammonium chloride	AA Wieral	1
Ascorbic acid	Ascorbic acid	3
Benzoic acid	Benzoic acid	3
Chlorium (cl)	Chlorin	4
Chlorine dioxide	Chlorin dioxide + DBSA	5
–	Meleusol	6
–	Obrivet	7
–	Trosilin liquid	6
–	Wofasept special	6
Ethyl alcohol	Ethanol	6
Formaldehyde	Formalin	6, 8
Isopropyl-alcohol	–	6
KMnO ₄	–	6
Nitrothal-isopropyl + captan	Pallicap 40%	1
Methylbromide	–	5
Paracetic acid	Wafasteril	6
p-Hydroxybenzoic acid	–	3
Propylene oxide	–	5
Sodium orthophenylphenate	Natriphene	1
Sodium orthophenylphenate	SOPP	3, 4
2-Bromo-2-nitropropane-1,3-diol	Bronopol 25%	1, 2
–	Fesia-form	1
–	Fesia-nom	1
B. Fungicides		
Bupirimate	Nimrod sp.	1
Captafol	Orthodifolatan 80	1
Imazalil	Fungaflor	1
Dodine	ICI Dodine	1
Dithianon	Delan 25%	2
Triadimefon	Bayleton 25	1, 2
Mancozeb	Dithhane M45, 80%	1
Benomyl	Benlate 50%	2
Quazatin triacetate	Befran 25	9
Hexachlorophene	Nabac 25	9
Zineb	Zinugec 80	2

continued

Table 11.3. *continued*

Common name	Commercial name	References
C. Various compounds		
Copper oxyquinolate		1
Garlic extract	Albarep	10
Hydroxyquinoline 20%		1
<i>Pingamia pinata</i> (extract)	Bactosan	11
Terpenes and terpenoids:		12
α -pinene, β -pinene, camphene, <i>d</i> -limonene, myrcene, α -terpinene, τ -terpinene, <i>l</i> -borneol, citral, dihydrocarveol, fenchone, isopulegol, linalool, dl-menthol, 2-pinanol, piperitone, pulegone, α -terpineol		
Experimental compounds	88201A	2
	8201B	2
	JF 4387	13, 14
	Fungex (liquid cuprammonium compound)	13
	Mixtures	
	Talosint (13.9% copper bis-extoxy, dihydroxy, diethyl, aminosulphate	2

DBSA, dodecyl benzene sulphononic acid.

References: 1. Koistra and Langeslang, 1981; 2. Paulin and Lachaud, 1984; 3. Janisiewicz and van der Zwet, 1988; 4. Sholberg *et al.*, 1988; 5. Roberts and Reymond, 1989; 6. Kleinhempel *et al.*, 1987; 7. Zeller *et al.*, 1984; 8. Beer and Rundle, 1987; 9. Tsiantos and Psallidas, 1993; 10. Sobiczewski and Berczynski, 1990; 11. Tsiantos and Psallidas, 1996b; 12. Scortichini and Rossi, 1991; 13. Gwynne, 1984; 14. Jones and Byrde, 1987.

1979). The active ingredient of these compounds is the copper ion, which is very toxic to all plant life. Copper ranks after silver (Ag) and mercury (Hg) for toxicity. Because of its high phytotoxicity, copper was not used as a foliar pesticide until 1885, when Millardet in France accidentally observed that a mixture of copper sulphate (CuSO_4) and lime (Ca(OH)_2) was not phytotoxic but exhibited a fungicidal action against *Plasmopara viticola* on grapevine. He then prepared the classical copper fungicide, called Bordeaux mixture, which later proved also to be a good bactericide. It has been used widely against bacterial diseases on different crops. The original Bordeaux mixture consists of 4.5 kg CuSO_4 and 5.5 kg Ca(OH)_2 in 454 l water. The chemistry of Bordeaux mixture and the precise mode of action are complex. The proportions of the ingredients used, and the method of preparation have considerable influence on the effectiveness of the product. In particular, the fineness and the composition control

Table 11.4. Bactericides registered for use against fire blight in different countries.

Common name	Commercial name	Registered for	Country
A. Copper compounds			
Ammoniacal copper sulphate	Copac E 40%	Apple ^b , Pear ^b	CY, BE ^c
Basic copper sulphate	Basicop	Apple ^b	US(NY)
Copper 20%	Burgundy mixture		
	(Burcor)	Apple ^a , Pear ^a	GR
Copper hydroxide	Not specified	Apple ^a , Pear ^a	BE, CY
	Blue shield DF	Apple ^a , Pear ^a	GR, NZ
	Champion	Apple ^a , Pear ^a	GR, NZ, BG ^d
	Kocide DF	Apple ^a , Pear ^a	NZ
	Kocide 101	Pear ^b	BG ^d , GR, US(NY)
	Kocide 2000	Apple ^a	US(NY)
	Cupravit	Apple ^a , Pear ^a	GR
	Funguran-OH 75WP	Apple ^a , Pear ^a	GR
	Parasol 50 WP	Apple ^a , Pear ^a	GR
Copper oxide	Nordox 50 WP	Apple ^a , Pear ^a	GR
Copper oxychloride	Not specified	Apple ^a , Pear ^a	BE, NL, CY
	Coperil	Apple ^a , Pear ^a	GR
	Copervall 50 WP	Apple ^a , Pear ^a	GR
	Cupranorg 35 WP	Apple ^a , Pear ^a	GR
	Cupravit OB-21 WP	Apple ^a , Pear ^a	GR
	Polvere cafarò	Apple ^a , Pear ^a	GR
Copper oxyquinolate	Quinololate 40%	Apple ^a , Pear ^a	CY
Copper oxychloride + mancozeb (46.1 + 15%)	Mankocide	Pear ^a	US(NY)
Copper oxychloride + maneb (37.5 + 20%)	Herkul	Pear ^b , Apple ^b	TR
Copper sulphate	Bordeaux mixture	Apple ^a , Pear ^a	GR, TR, BE ^c , BG ^d
Tribasic copper sulphate	—	Apple ^a , Pear ^a	CY
Various copper compounds	Not specified	Apple ^a , Pear ^a	CA, US, DE
		Ornamentals ^{a,b}	FR
B. Antibiotics			
Oxytetracycline	Mycoshield	Pears ^b	US(MI), (UT)
	Terramycin	Pears ^b , Apple ^b	US(WA)
Streptomycin	Not specified	Apple ^b , Pear ^b	CA, IL
	Agrimycin R17	Apple ^b , Pear ^b	NZ, US
	Plantomycin	Apple ^b , Pear ^b	NL, IL
	Fructocin 17%	Apple ^b , Pear ^b	BE
	Agrept	Apple ^b , Pear ^b	GR
Streptomycin + oxytetracycline	Bacterol super	Apple ^b , Pear ^b	GR
Kasugamycin	Kasumin	Ornamentals ^b	NL
C. Other compounds			
Flumequin	Firestop TM	Apple ^b , Pear ^b	BE ^c , FR, CY
Fosetyl-Al	Aliette TM	Apple ^b , Pear ^b	FR, TR
Oxolinic acid	Starner TM 20%	Apple ^b , Pear ^b	IL

^a During dormancy, before bloom.^b During blooming.^c Registered but not used.^d Not registered specifically for fire blight but used since it has been registered for other diseases on pears and apples.

Country codes according to ISO 3166 : 1988.

the physical properties of the dry deposit on the plant surfaces, which in turn greatly affect its tenacity and activity (Gremlyn, 1990). Since the soluble copper compounds are too phytotoxic to be used as foliar sprays, different insoluble ones, besides Bordeaux mixture, have been employed. The most important are copper hydroxide, copper oxychloride and cuprous oxide, which have the advantage of being easier to prepare and to apply than Bordeaux mixture.

The activity of the copper compound is due to the formation of soluble copper by any of the three following main agents:

1. Atmospheric carbon dioxide and ammonium salts dissolved in rainwater or dew.
2. Microbial secretions on the plant surface.
3. Secretions from the healthy or wounded surface of the host plant.

Both microbial and plant exudates contain certain amino acids and hydroxy acids that can chelate copper, which then dissociates in solution, yielding toxic cupric ions. Durkee (cited by Martin and Woodcock, 1983) found that copper compounds that exhibited a fungicidal activity were lipid-soluble, so it may be suggested that the function of exudates on the plant surface is to provide for the formation of a lipid-soluble copper complex that can penetrate the pathogen's cell wall. Dissociation of the complex within the bacterium will give rise to cupric ions, which may interfere with cell metabolism, causing cell death or inhibiting bacterial biological activities. The copper ions are also toxic to plant cells, causing damage to leaves and fruits. On leaves, necrotic areas start as small purple flecks. On fruits, the killing of the epidermis at localized areas often results in cork formation, producing a 'russetting' on the surface of the fruit, which affects its commercial value.

Bordeaux mixture (copper sulphate + lime) is still in use and it is the standard against which other compounds are evaluated. The most frequently used Bordeaux mixture formula is 1-1-100 (1 kg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 kg Ca(OH)_2 in 100 l water), which contains 250 g Cu hl^{-1} . This concentration alone or with 1% spray oil (van der Zwet and Beer, 1992) is recommended for pre-bloom or bud break, as well as autumn sprays (Garrett, 1990). Concentrations of 50-100 g Cu hl^{-1} are recommended for the blossom period (Garrett, 1990).

The main disadvantage of Bordeaux mixture is its phytotoxicity on some host plants, especially pears. Attempts to overcome phytotoxicity by adding more lime had no effect; furthermore, an excess of lime may itself be phytotoxic to certain plants (Martin and Woodcock, 1983). Reduction in phytotoxicity is obvious when the copper concentration is lowered, but the efficacy is also altered and insufficient control is achieved, especially under high inoculum pressure.

In order to overcome the phytotoxicity of Bordeaux mixture and to facilitate the preparation and application of sprays, different formulations of copper have been tested (van der Zwet and Keil, 1979; see Table 11.1). Most of the copper formulations were equal to or better than Bordeaux mixture against fire blight. Koistra and de Gruyter (1984), testing copper oxychloride and

ammoniacal copper sulphate, concluded that both formulations were equally effective against flower infection, provided that the amount of copper applied is the same. The most frequently used and commercially available copper formulations are: ammoniacal copper sulphate, copper hydroxide, copper oxide, copper oxychloride and mixtures of copper compounds with maneb, zineb and oil. All copper formulations, with artificial or natural infections, were equally or more effective against *E. amylovora* than Bordeaux mixture and some were comparable to streptomycin (see Table 11.1). However, all these products are phytotoxic at the doses recommended for efficient fire blight control during bloom and post-bloom.

Mixtures of copper compounds with other chemicals have also been tested. Tri Miltox-forte (three copper salts + maneb) gave promising results (Koistra and de Gruyter, 1984; Paulin and Lachaud, 1984; Saygili and Üstün, 1996) and Sobra spray (copper sulphate + foliar fertilizers) gave medium control of blossom and shoot blight on apples (Clarke *et al.*, 1993), while Cuivrol (copper sulphate 18% + oligoelements) gave satisfactory results (Larue and Ardigier, 1993) on apple blossom without phytotoxic effect at a dose of 50 g Cu hl⁻¹. Kasumin-Bordeaux (kasugamycin 5% + copper oxychloride 45%) proved phytotoxic at a dose of 300 p.p.m. a.i. of kasumin (Bonn, 1984).

Although resistance of *E. amylovora* to copper has not yet been detected, we cannot rule out that it will develop, since resistance to copper has been reported in other phytopathogenic bacteria. Copper resistance is widespread among *Xanthomonas campestris* pv. *vesicatoria*, where it is conferred by a 200 kb self-transmissible plasmid, pXvCu (Cooksey, 1990), while in *Pseudomonas syringae* pv. *tomato* resistance is determined by a 35 kb plasmid (pPT23D), which is not self-transmissible but can be mobilized. Copper resistance has also been found in *P. syringae* pv. *syringae* (Sundin *et al.*, 1989). The resistance in these *Pseudomonas* strains is mediated by a 61 kb plasmid, which is transferable to strains of *P. syringae* but not to strains of *P. syringae* pv. *mors-prunorum*. The same is true for the pXcCu plasmid, which was never transferred to other plant-pathogenic bacteria (Bender *et al.*, 1990). The mechanisms of copper resistance have been reviewed by Cooksey (1990).

Antibiotics

Antibiotics are organic compounds produced by microorganisms which selectively inhibit the growth of other microorganisms. The use of antibiotics against bacterial pathogens of cultivated plants started in 1950, after the successful use of antibiotics against difficult-to-control human diseases. Fire blight was among the first bacterial diseases for which control by antibiotics was tested, since the use of copper compounds is restricted because of phytotoxicity. *E. amylovora* is sensitive to many antibiotics *in vitro* (Paulin, Chapter 6). Morgan and Goodman (1955) found that in the laboratory aureomycin, neomycin, streptomycin, polymyxin, streptotrichin, viomycin and chloromycetin were effective against

E. amylovora. Rudolph (1946) found that penicillin inhibited *E. amylovora* *in vitro* but that it failed to control fire blight in field experiments. Later, Billing *et al.* (1961) reported that *E. amylovora* was resistant to penicillin *in vitro*. Martinec and Kocur (1964) found that all 49 strains of *E. amylovora* tested were sensitive to chloramphenicol, erythromycin, neomycin, streptomycin and tetracycline, while 24 were sensitive to chlortetracycline. All strains were insensitive to penicillin, tyrothricin, nystatin and bacitracin. In field experiments, Paulin and Lachaud (1984) reported that polyoxin (Laicon) failed to control *E. amylovora* on *Cotoneaster* sp. flowers. Both laboratory and field experiments (Kloss, 1969) indicated that spectinomycin significantly controlled fire blight.

Although many antibiotics inhibit growth of *E. amylovora* *in vitro*, only a few have practical value for field applications, because of plant or mammalian toxicity, lack of systemic activity and the short persistence on the plant surfaces under field conditions (Ark, 1949). Of the large number of antibiotics evaluated against fire blight, only streptomycin and, to some extent, oxytetracycline and kasugamycin have met the necessary requirements to be used in field applications.

Streptomycin

Streptomycin is an aminoglycoside produced by certain strains of *Streptomyces griseus*. It was discovered by Schatz *et al.* in 1944 and used successfully against the bacterium responsible for tuberculosis (*Mycobacterium tuberculosis*). The prime effect of streptomycin is on protein synthesis. It limits the normal growth of the cells by causing them to misread the genetic code from messenger RNA. Because of this, abnormal enzymes might be produced, leading to the malfunction and death of the cell (Gottlieb and Shaw, 1970). On higher plants, it inhibits chlorophyll synthesis, resulting in chlorosis of the leaves. Streptomycin is easily taken up by the roots of the plants, but its concentration in plant tissues has no effect on subsequent inoculation with bacterial plant pathogens (Anderson and Nienow, 1947). High concentrations of streptomycin may cause the chlorosis and death of the plants.

After its successful use against tuberculosis and the observation that it probably moves systematically into plant tissues, streptomycin was tested against different plant-pathogenic bacteria *in vitro* and *in vivo*. Ark (1947) reported that streptomycin inhibited 14 species of plant-pathogenic bacteria, both Gram-positive and Gram-negative. Early experiments (Heuberger and Poulos, 1953; Ark and Scott, 1954) showed that streptomycin applied at 30, 60, 120 and 240 p.p.m., respectively, controlled fire blight efficiently without any phytotoxic effect on the leaves and without causing fruit russetting. Dunegan *et al.* (1954) reported effective control of fire blight on pear by a streptomycin–oxytetracycline mixture. After these encouraging reports, a large number of experiments were performed (van der Zwet and Keil, 1979), which proved that streptomycin presented a good weapon to fight the disease. The rate of 100–150 p.p.m., applied during the blooming period at 3–5-day intervals,

gave good control. In cases where secondary blossoms develop or the weather conditions favour infections, more applications are recommended (Bonn and Morand, 1980). Since streptomycin has limited systemic activity it should cover all the sites vulnerable to infection, e.g. open flowers, shoot or leaf. Van der Zwet and Keil (1972) reported that streptomycin sprays within 6 h after inoculation of injured tissues effectively protected the injured pear trees, while uninjured trees were protected for 4 days.

Although streptomycin is considered the most effective bactericide against fire blight, with no real phytotoxic problems at the recommended rates, its use in agriculture has been prohibited in many countries. The main reason for this is the development of resistance to streptomycin not only by *E. amylovora* but also by other microorganisms on the plant surface or in the soil or water, including possible human or veterinary pathogens (Jones and Schnabel, Chapter 12). The problem of streptomycin-resistant mutants of the target microorganism was encountered shortly after the use of streptomycin to control tuberculosis (Steenken and Wolinsky, 1949). The pathogen rapidly developed highly resistant strains both *in vitro* and in treated patients.

English and van Helsema (1954) reported that the resistance to streptomycin of two *E. amylovora* strains increased by 250 to 500 times after 13 transfers on media containing the antibiotic. The authors demonstrated also that the combination streptomycin + 10% oxytetracycline prevented or delayed the emergence of streptomycin-resistant mutants. Streptomycin-resistant *E. amylovora* strains in orchards were first reported in California in 1972 (Miller and Schroth, 1972; Moller *et al.*, 1972). Since then, streptomycin-resistant *E. amylovora* strains have been found in many places where streptomycin sprays are used to control fire blight (Jones and Schnabel, Chapter 12). Streptomycin resistance has been reported in Oregon and Washington (Coyier and Covey, 1975), Missouri (Shaffer and Goodman, 1985) and Michigan (Chiou and Jones, 1991) and, outside the USA, in Egypt (El-Goorani *et al.*, 1989) and in New Zealand (Thomson *et al.*, 1993).

Mechanisms used by bacteria to cope with streptomycin include alterations of the ribosomal target site, production of streptomycin-modifying enzymes and prevention of streptomycin access to the target site (Amyes and Gemmell, 1992). The genetic basis for streptomycin resistance in *E. amylovora* has been extensively studied (Schroth *et al.*, 1979; Chiou and Jones, 1991, 1993, 1995; McManus and Jones, 1994; Jones and Schnabel, Chapter 12). It has been found that two phenotypes, highly resistant (HR) and moderately resistant (MR) to streptomycin, exist among the populations of *E. amylovora* and that, in the HR strains, resistance is attributed to mutation in the *rpsL* gene, while, in MR strains, resistance is attributed to the presence of acquired plasmid or transposon genes coding for the degradative enzyme, streptomycin-phosphotransferase, thus inactivating streptomycin. Such a distinction is not true for strains isolated from New Zealand (Vanneste and Voyle, 1999). The chromosomal mutation resistance is not transferable by conjugation, while the plasmid-borne resistance is transferable. It has also been demonstrated (Minsavage *et al.*, 1990; Jones *et*

al., 1991; Norelli *et al.*, 1991; Burr *et al.*, 1993) that the plasmid-borne gene for streptomycin resistance is widely distributed geographically in *P. syringae* pv. *papulans* and in *X. campestris* pv. *vesicatoria* and among a diverse group of Gram-negative bacteria isolated from apple orchards. Chiou and Jones (1991) demonstrated that a high frequency of transfer of streptomycin resistance exists between the donor and recipient strains when the resistance is plasmid-mediated. This may result in a rapid increase in streptomycin-resistant strains in the orchard.

Because of the problem of the development of resistance to streptomycin and/or its transfer among diverse bacterial populations, its use has been entirely prohibited for field sprays in many European countries. There are some special regulations in certain countries, such as Belgium, Germany, Greece, Israel and The Netherlands, where streptomycin is allowed for field sprays against *E. amylovora* only during the primary blooming period.

Oxytetracycline

Oxytetracycline is an antibiotic produced by the actinomycete, *Streptomyces rimosus*; it is sold under the name Terramycin. Oxytetracycline is a protein synthesis inhibitor that interferes with the binding of tRNA to the ribosome. It has been tested against fire blight, on its own or in combination with streptomycin, since antibiotics have been used to control fire blight.

Winter and Young (1953) reported that oxytetracycline at 120 p.p.m. was less effective than streptomycin, while Heuberger and Poulos (1953) found at the same concentration that it was ineffective against fire blight. Dunegan *et al.* (1954) found that a mixture of oxytetracycline and streptomycin at a dose of 100 and 10 p.p.m., respectively, effectively controlled fire blight. Oxytetracycline has been used since the late 1970s to control fire blight in the western USA (Moller *et al.*, 1981). It has also been approved by the Environmental Protection Agency (EPA) for use in Michigan, where streptomycin resistance has been confirmed (McManus and Jones, 1994). Moller *et al.* (1972) reported that oxytetracycline had been effective in orchards where streptomycin had failed and that it was less phytotoxic than copper. McManus and Jones found that oxytetracycline was inferior to streptomycin in controlling colonization of stigmatic surfaces by a streptomycin-sensitive strain, but it was superior to streptomycin when a streptomycin-resistant strain of *E. amylovora* was used. They explain this behaviour of oxytetracycline on the basis that streptomycin is bactericidal while oxytetracycline is bacteriostatic. The authors recommend the use of oxytetracycline for fire blight control in areas where streptomycin-resistant strains of *E. amylovora* exist. Although resistance to oxytetracycline has not been found in *E. amylovora* (Loper and Henkels, 1991) and its presence in a mixture with streptomycin delays the emergence of streptomycin-resistant strains of *X. campestris* pv. *vesicatoria* and *E. amylovora* (English and van Halsema, 1954), the intensive use of this antibiotic could potentially result in the emergence of oxytetracycline-resistant strains of *E. amylovora*. There are several reports (Lee

et al., 1993) indicating that tetracycline resistance is widespread among bacterial species from the faeces and intestinal tracts of swine. As in streptomycin resistance, the determinants of resistance to tetracycline are carried on chromosomes, on plasmids or on both, and the levels of tetracycline tolerated by the resistant bacteria depend on the type of determinant (Jones and Schnabel, Chapter 12). Chromosome-conferred resistance is higher than plasmid-conferred resistance (Lee *et al.*, 1993).

Formulations containing approximately 15% streptomycin and 1.5% oxytetracycline (Agrimycin-100, Bacterol super, etc.) were usually used against fire blight during the 1950s to prevent the development of streptomycin resistance in *E. amylovora*. Later, oxytetracycline was removed, probably because the combination showed no immediate advantage when used in the field.

Kasugamycin

Kasugamycin is an aminoglycoside produced in the culture broth of *Streptomyces kasugaensis*. It has been developed against rice blast, a fungal disease of rice, caused by *Piricularia oryzae*. Although it has been developed as a fungal antibiotic, it proved to be a good bactericide as well. Kasugamycin is traded under the name Kasumin antibiotic. The antibiotic inhibits protein synthesis at low concentrations and acts by preventing the incorporation of amino acids by inhibiting the binding of the aminoacyl-tRNA-message complex and the ribosome. It does not cause misreading of the genetic code, as streptomycin does (Coster and Waalkens, 1989). The antibiotic has been tested against fire blight at doses of 50–500 p.p.m. with various results, ranging from no significant control (Paulin and Lachaud, 1984; Paulin *et al.*, 1987) to medium control (Tsiantos and Psallidas, 1996b) or good control (Koistra and de Gruyter, 1984; Coster and Waalkens, 1989; Aldwinckle and Norelli, 1990; Saygili and Üstün, 1996; Tsiantos and Psallidas, 1996b). Its use is restricted because of its high phytotoxicity on pears and apples at the recommended doses for effective control of fire blight (Bonn, 1984; Koistra and de Gruyter, 1984; Aldwinckle and Norelli, 1990). However, Kasumin has been registered in The Netherlands for use on ornamental hosts for fire blight control, since no phytotoxicity problems occurred (Koistra and de Gruyter, 1984; Coster and Waalkens, 1989), at a dose of 300 p.p.m.

Other compounds

Flumequin

Flumequin is a synthetic, non-antibiotic, non-sulphamide bactericide, active against both Gram-negative and Gram-positive bacteria. It has been marketed as FirestopTM, FructilTM and MBR 10995. Its chemical name is (1H–5H)-dihydro-6,7- fluoro-9-methyl-5-oxo-1-benzo-(I, j)-quinolizin carboxylic acid-2.

Flumequin blocks DNA replication by interfering with the DNA gyrase, the enzyme required for DNA supercoiling. DNA gyrase is also required for the maintenance and transfer of 'R'-type plasmids. Plasmid-borne resistance to the chemical has never been observed. The chemical has been tested in different countries against fire blight on different hosts (see Table 11.1) and has given promising results. Consequently, it has been registered for fire blight control on apples, pears and ornamental crops in France, Belgium and Cyprus, and has been recommended for control of fire blight at a rate of 300 p.p.m. a.i. hl^{-1} sprayed during the flowering period. The number of applications varied, depending on the infection risks during blooming. The chemical has no systemic activity and has not been reported to produce any phytotoxic symptoms.

Fosetyl-aluminium (fosetyl-Al)

Fosetyl-Al is an acidic systemic fungicide marketed under the name AlietteTM and used primarily against different species of the genus *Phytophthora*. However, it has been found to be effective against other phycomycetes, as well as other phytopathogenic fungi. It has been reported to give systemic control of downy mildews of vines, tropical crops and vegetables. Early reports on the mode of action of fosetyl-Al suggest that the compound does not directly inhibit the target organisms (Farih *et al.*, 1981; Guest, 1984), but that it induces defence mechanisms in the host. When applied either as foliar sprays or in the root system, fosetyl-Al moves systemically into the plant and triggers its defence mechanisms, thus preventing infection (Guest, 1986). However, new evidence supports the hypothesis that fosetyl-Al exhibits both direct and indirect activity against the target organisms (Fenn and Goffey, 1984, 1985; Guest, 1986). Fosetyl-Al, or its metabolite, phosphonic acid, probably acts directly on the target pathogen, slowing its growth, allowing the host plant's natural defence mechanisms to finally inhibit its growth.

The action of fosetyl-Al against bacterial plant pathogens also appears to be both direct and indirect (Chase, 1993). *E. amylovora* tolerated *in vitro* concentrations as high as 1000 mg a.i. l^{-1} . However, because of its systemic property, fosetyl-Al was thought to be a good bactericide against fire blight. The results of experiments conducted in both the USA and Europe (see Table 11.1) showed that its effectiveness was inconsistent. Norelli and Aldwinckle (1993) reported that the use of AlietteTM gave poor control of fire blight under both high or low inoculum pressure and it failed to control blossom blight in spring when applied the previous autumn. Similar results have been reported by Clarke *et al.* (1993), while a significant difference in blighted shoots was found, especially when a high dose (1200 p.p.m.) was applied. Tsiantos and Psallidas (1993a, b, 1996b) found no significant effect of fosetyl-Al in either preventive or curative sprays with artificial inoculations, while with natural infections a significant effect of AlietteTM was observed, probably because of low inoculum pressure (Tsiantos and Psallidas, 1996a). At a dose of 300 g hl^{-1} , it showed some phytotoxicity. Burr and Norelli (1984) reported effective control of fire blight with

fosetyl-Al, equal to that of streptomycin. No significant difference in dose effect was observed, probably because of unfavourable climatic conditions for disease development. Paulin *et al.* (1990) reported that Aliette™ had been useful in controlling fire blight in the orchard at a dose greater than 2 g a.i. l⁻¹. Its effectiveness was comparable to that of streptomycin, but results were inconsistent. No curative effect had been noticed.

Larue and Gailliard (1993) reported that Aliette™ at 3 g a.i. l⁻¹ diluted in a water spray volume of 1000 l ha⁻¹ was very effective in preventing fire blight occurrence in both pear and apple orchards. The best results were obtained when the pesticide was applied just before the infection. The applications should be repeated every 3 days, depending on the infection risk (temperature > 24°C or 21°C maximum and 12°C minimum). The authors also recommend immediate intervention after thunderstorms or hail.

Fosetyl-Al has been registered for fire blight control in France and Turkey.

Comparing the results obtained by different investigators in controlling fire blight with fosetyl-Al with the results obtained from experiments against other bacterial diseases, it seems that there are difficulties in obtaining consistent results, not only from year to year but also from trial to trial. Because of this inconsistency, Chase (1993) does not recommend fosetyl-Al to be used for control of bacterial diseases on ornamental crops.

Oxolinic acid

Oxolinic acid is a relatively new synthetic bactericide, of the quinoline family, developed by Sumitomo Chemical Co. (Hikichi *et al.*, 1989). Oxolinic acid, according to manufacturers, exhibits both preventive and curative activities against plant diseases caused by bacteria belonging to *Pseudomonas* spp. and *Erwinia* spp. Its chemical structure is 5-ethyl-5,8-dihydro-8-oxo-(1,3)-dioxolo-(4,5g)-quinoline-7-carboxylic acid. It was registered against rice seedling rot caused by *P. syringae* pv. *glumae* in Japan in 1988 under the trade name Starner™. It has also been released with the code no. S-0208 for experimental use.

Its mode of action has not been reported so far. Oxolinic acid (S-0208) was tested against fire blight in different countries during the 1980s. Paulin and Lachaud (1984) reported rather poor results, while Jones and Byrde (1987) obtained excellent (99%) control of fire blight on cider apples by twice-weekly protective sprays at 300 p.p.m. a.i. hl⁻¹. Excellent results have also been obtained by Deckers *et al.* (1990) in Belgium, by Dimova-Aziz (1990) in Cyprus and by Tsiantos and Psallidas (1993a, b, 1996b) in Greece. No case of phytotoxicity was observed on either pears or apples. In most cases, oxolinic acid was used as preventive treatment during the blossom period, but some curative effects were also observed (Tsiantos and Psallidas, 1993a, b, 1996b).

CGA 73089

CGA 73089 (7-chloro-1-ethyl-6-fluoro-1,4-dihydro-4-exo-3-quinoline carboxylic acid), produced by Ciba Geigy, was incorporated in many trials against

fire blight in the 1980s (see Table 11.1) and gave promising results. Unfortunately, it had to be withdrawn because of unacceptable levels of mammalian toxicity.

Disinfectants

Epidemiological studies by van der Zwet and Keil (1979) showed that fire blight can be spread from infected trees to healthy ones in the orchard or to other orchards by pruning tools, hands and boots and over long distances by infected packing boxes or contaminated plant material.

To prevent such spread, disinfection of pruning tools, hands and boots, as well as packing boxes and plant material, including fruits, is generally recommended. However, there have been only a few studies to determine the efficacy of disinfectants on *E. amylovora*. Furthermore, in some cases, the results reported are contradictory. In general, for cutting shears, dipping in ethanol 70% (Beer and Rundle, 1987; Hasler *et al.*, 1996), isopropanol 70% (Beer and Rundle, 1987), sodium hypochlorite (NaOCl) (Hasler *et al.*, 1996) or Lisetol (Billing, 1983; Hasler *et al.*, 1996) has given satisfactory results. Hot-water treatment has a 100% effect after 5 min incubation time, but it is difficult to organize such treatment in the field (Hasler *et al.*, 1996). Although sodium hypochlorite has a very good and rapid effect, its use has some drawbacks since it is corrosive and irritant. Lisetol seems to be the most effective and easy-to-use disinfectant (Billing, 1983; Hasler *et al.*, 1996). For disinfection of hands, good results have been obtained by the clinical disinfectants Sagrosept and Sterillium (Hasler *et al.*, 1996).

Deckers *et al.* (1987b) reported that, although several disinfectants killed *E. amylovora* *in vitro* after 5 min contact, only 50% sodium hypochlorite or concentrated Dettol killed high concentrations of the pathogen on contaminated knives. The authors recommended Dettol, because it is less corrosive than sodium hypochlorite.

For the disinfection of pear and apple fruits, the best results have been obtained by dipping for 10 min in a citrate buffer 0.1 M (2.5 pH) or 250 p.p.m. NaOCl in 500 p.p.m. (DBSA) (Roberts and Reymond, 1989) or 500 p.p.m. NaOCl in 0.25% Ortho X-77 (Janisiewicz and van der Zwet, 1988).

A 10 min dipping in 1M acetic or propionic acid was found to eliminate *E. amylovora* from the surface of apple fruits without any phytotoxicity symptom (Sholberg *et al.*, 1988). However, Roberts and Reymond (1989) consider that treatment with 1 mol l⁻¹ acetic or propionic acid is not realistic, since these acids are associated with strong, pungent vapours, which are noxious and might cause worker discomfort in the enclosed environment of the packing house.

Janisiewicz and van der Zwet (1988) reported that benzalkonium chloride at 1400 mg l⁻¹ or 2000 p.p.m. + 2500 p.p.m. Ortho X-77 (Roberts and Reymond, 1989) has given promising results in eliminating *E. amylovora* from apple fruits; however, neither benzalkonium chloride nor the surfactant Ortho

X-77 has been registered for use in food products (Roberts and Reymond, 1989).

Plant extracts

Plant extracts have been tested against *E. amylovora* *in vitro* and *in vivo* (see Table 11.3). Scortichini and Rossi (1989) found that essential oils from origanum, garlic, savory, camomile and white thyme exhibited antibacterial activity against *E. amylovora* *in vitro*. They reported that there are some differences in sensitivity among different isolates of the bacterium. Mosch *et al.* (1989) reported that 24 out of 131 plant extracts tested exhibited some degree of antibacterial activity against *E. amylovora*, the most effective being leaf extracts from *Rhus typhina*, *Jughans nigra*, *Berberis vulgaris*, *Mahonia aquifolium* and extracts from *Alium sativum*. Vanneste (1996) also reported that some plant extracts and some essential oils could inhibit *E. amylovora* *in vitro*, in particular thyme oil, cinnamon oil and α -terpineol.

Field experiments with extracts from *M. aquifolium*, *B. vulgaris*, *R. typhina* and *A. sativum* as protective sprays did not show satisfactory disease control (Mosch *et al.*, 1990). Mosch *et al.* (1993) demonstrated that plant extracts from *Reynutria sachalinensis*, *Hedera helix*, *Viscum album* and *Alchemilla vulgaris* could induce resistance mechanisms in detached leaves of *Cotoneaster watereri*, thus reducing the multiplication of the bacterium in the leaf tissues, resulting in lowering the disease severity. The same results have been reported with plant extracts from *H. helix* and *V. album* on detached leaves of *Cydonia oblonga*. In field experiments on apple blossom of 'James Grieve', *H. helix* extract gave a comparable level of control to that of streptomycin (Mosch *et al.*, 1996). In these experiments it was found that some physiological changes occurred in the plant tissues, such as increased activities of chitinase, β -1,3-glucanase and enzymes of phenol metabolism, which can be regarded as a marker of the induction of resistance. Tsiantos and Psallidas (1996b) found that Bactosan, an extract from *Pingania pinata*, showed some effect on fire blight on blossom in field experiments. Scortichini and Rossi (1989, 1991, 1993), studying the influence of some essential oil constituents on bacterial growth, observed that the terpenoids geraniol and citrolleol, out of 20 terpenes and terpenoids tested, exhibited bactericidal activity against *E. amylovora* at a concentration of 1500 mg l⁻¹. They also reported that, of these two terpenoids, citrolleol was less effective than geraniol, being bactericidal for only two of seven strains tested.

Recommendations

Chemical treatments are very useful and play an important role in the effort to eliminate losses in fruit production due to infection by *E. amylovora*. However, no complete protection could be expected by chemical treatments alone. The

Table 11.5. Recommended spray schedule for fire blight control.

Time of spray application	Chemical	Concentration
Pre-bloom after the swollen bud stage but before bud break	Bordeaux mixture + 1% spray oil or copper oxychloride + oil 1% copper hydroxide + oil 1% or copper oxychloride sulphate + oil 1% (COCS)	250 g Cu hl ^{-1a}
Blossom period ^b	Copper compounds (Bordeaux mixture, copper oxychloride, etc.)	50–100 g Cu hl ⁻¹
	or flumequin (Firestop TM , Fructil TM)	300 p.p.m.
	or fosetyl-Al (Aliette TM)	3000 p.p.m. (0.3 kg hl ⁻¹)
	or oxolinic acid (Starner TM)	300 p.p.m.
	or streptomycin (Plantomycin, Agrept, Agristrep)	100 p.p.m.
	or oxytetracycline (Mycoshield)	200 p.p.m.
	or streptomycin + oxytetracycline (Bacterol super)	100 p.p.m.
Summer (after storms) ^c	The same as in blossom period	
Autumn ^d	Copper compounds preferably Bordeaux mixture	250 g Cu hl ⁻¹

^a 2500 l ha⁻¹ to run off should be applied (van der Zwet and Beer, 1991).

^b At 3–5-day intervals, or according to the prediction system's recommendation (van der Zwet *et al.*, 1988).

^c Bactericides should be applied within 24 h after the storm or if possible immediately after the storm (van der Zwet and Beer, 1991).

^d Two copper sprays during leaf fall in orchards where fire blight has occurred are recommended to reduce the number of active cankers.

chemicals should be considered as a part of an integrated control system aiming to reduce fire blight incidence (Steiner, Chapter 17). The timing of chemical sprays during the periods of susceptibility to infection should be the main concern of the growers and advisers, since it is very important both for the efficacy of sprays and the optimization of applications. This may result in savings for the grower, as well as preventing environmental problems.

There are several prediction systems, such as MaryblytTM, Powell, Fire-screens and the Billing revised system, which can help to evaluate the necessity of spraying (see Billing, Chapter 15).

The research for new chemicals against *E. amylovora* over the last 15 years has resulted in the release of new compounds, which give promising results and can be used against fire blight, replacing copper compounds or streptomycin, which cannot be used either because of phytotoxicity and resistance problems or because they are not allowed to be used. Some promising compounds, which have been registered in some countries, are flumequin (FirestopTM, FructilTM), fosetyl-Al (AlietteTM) and oxolinic acid (StarnetTM). Other compounds include BTH (Novartis Crop Protection) (BionTM, ActigardTM), prohexadione-Ca (ApogeeTM) and harpin (MessengerTM) which, however, have not been registered in any country for field use.

In conclusion, a spray schedule for the chemical control of fire blight may be recommended, only as a guideline (Table 11.5). It should, in any case, be adapted according to the prevailing conditions and the chemicals and biological control agents (Johnson and Stockwell, Chapter 16) registered for fire blight control in a given place.

For the disinfection of contaminated pruning material, shears, saws, knives, etc., dipping in 70% ethanol for 10 min, Lisetol 4% or Dettol (concentrated) is recommended. For the disinfection of hands, washing with ethanol 70%, Sagrosept or Sterillium is effective. Spraying boots with ethanol 70% for 1 min eliminates *E. amylovora* cells. Finally, the disinfection of pear and apple fruits could be accomplished by dipping in 0.1 M citrate buffer (pH 2.5) plus 500 p.p.m. DBSA or NaOCl 250 p.p.m. plus 200 p.p.m. surfactant (Ortho X 77).

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The Development of Streptomycin-resistant Strains of *Erwinia amylovora*

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Introduction

Streptomycin, an aminoglycoside antibiotic discovered in 1944 (Schatz *et al.*, 1944), was shown to exhibit fire blight control activity in 1952 (Murneek, 1952; Heuberger and Poulos, 1953) and was first registered as a pesticide in the USA in 1955. The efficacy of bloom applications for control of the blossom blight phase of the disease was established in numerous orchard trials conducted throughout the USA in the 1950s and 1960s. By the late 1960s, the practice of using streptomycin for fire blight control on apples and pears was well established in North America. In the high-value pear-growing areas of the western USA, streptomycin was used in pre-bloom, bloom and post-bloom applications up to 30 days before harvest (90 days before harvest prior to about 1968). In apple-growing areas of the eastern USA, streptomycin was used in bloom applications and occasionally in post-bloom applications up to 50 days before harvest (90 days prior to about 1968). It continues to be used throughout North America for fire blight control and is either applied alone or mixed with oxytetracycline. Streptomycin is also used for fire blight control in New Zealand and recently in some European and Middle Eastern countries (Psallidas and Tsiantos, Chapter 11).

Strains of the phytopathogenic bacterium that causes fire blight, *Erwinia amylovora*, that are resistant to streptomycin have been detected in several areas of the USA. The first report was of strains resistant to streptomycin isolated from pear orchards of California in 1971 (Miller and Schroth, 1972). It was followed by a report of resistance in strains isolated from Washington and from Oregon (Coyier and Covey, 1975). Today, streptomycin-resistant strains are widespread in apple and pear orchards of the western USA (Loper *et al.*, 1991; Stockwell *et*

al., 1996). Contrary to the situation in western states, most apple orchards in eastern North America, even after more than 40 years of streptomycin usage, contain only streptomycin-sensitive strains of *E. amylovora*. Several attempts to detect resistance in Michigan in the mid-1970s (Sutton and Jones, 1975) and in New York in the mid-1970s (Beer and Norelli, 1976) and again in 1991 (Burr *et al.*, 1993) were unsuccessful. Detection of resistance in Missouri apple orchards in 1985 provided the first report of resistance in the eastern USA (Shaffer and Goodman, 1985). Eventually, in 1990, a second outbreak of streptomycin-resistant strains of *E. amylovora* was detected in an apple orchard in Michigan (Chiou and Jones, 1991) and later in two other areas in Michigan (McManus and Jones, 1994). Even in Michigan, the majority of the apple orchards contained only streptomycin-sensitive strains (McManus and Jones, 1994). Recently, streptomycin-resistant *E. amylovora* were found outside North America in New Zealand (Thomson *et al.*, 1993) and Israel (Manulis *et al.*, 1996).

The existence of streptomycin-resistant *E. amylovora* makes control difficult, if not impossible, because streptomycin is the only effective, plant-safe pesticide available in many countries for the control of fire blight (Psallidas and Tsiantos, Chapter 11). The less effective antibiotic oxytetracycline can be substituted for streptomycin or used in combination with streptomycin on pears in the USA. Oxytetracycline is used to control fire blight of apple in areas of the USA with streptomycin-resistant *E. amylovora*; this use for oxytetracycline must be approved annually. Streptomycin is also used to complement microbial control of the blossom blight phase with *Pseudomonas fluorescens* strain A506 (Johnson and Stockwell, Chapter 16). The need to maintain the effectiveness of streptomycin is increasing as fire blight expands geographically into new areas and as plantings of high-value blight-susceptible apple cultivars and rootstocks increase worldwide (Lespinasse and Aldwinckle, Chapter 13).

The recovery of streptomycin-resistant strains of *E. amylovora* in pear orchards in California in 1971 and in Michigan in 1990 stimulated research on the nature and development of streptomycin resistance in phytopathogenic bacteria. These studies have contributed to the body of knowledge on the development, detection and nature of streptomycin resistance in *E. amylovora*. In this review, we describe the mechanisms by which streptomycin resistance can evolve and provide suggestions on how to delay problems with resistance in those areas where it is currently not a problem.

Genetics of resistance

Streptomycin resistance in bacteria can occur either as a result of chromosomal mutation or through gene acquisition (Davies, 1986). Streptomycin acts by binding to the bacterial ribosome and inhibiting protein synthesis. All mutations that result in streptomycin resistance analysed thus far are point mutations in ribosomal genes, which alter the streptomycin target site, thus preventing the binding of streptomycin to the ribosome. Acquired resistance results

from the transfer of streptomycin resistance genes from one organism to another. The acquired genes enable bacteria to produce aminoglycoside-modifying enzymes, which alter the streptomycin molecule such that it is unable to bind to the ribosome.

Determination of the minimum inhibitory concentration (MIC) is the most widely used method for the initial characterization of streptomycin-resistant strains. Two streptomycin-resistant phenotypes, highly resistant (HR) and moderately resistant (MR), are commonly identified among resistant field strains of *E. amylovora* (Coyier and Covey, 1975; McManus and Jones, 1994; Chiou and Jones, 1995b). The level of resistance to streptomycin is significantly higher in strains of *E. amylovora* with a point mutation than in strains with acquired resistance (McManus and Jones, 1994; Chiou and Jones, 1995b). Therefore, the genetic mechanism for resistance can be predicted based on the concentration of streptomycin required to inhibit the growth of resistant strains. In addition, insensitivity to mycomycin, an antibiotic produced by *Nocardia* sp. that resembles streptomycin in biological activity (Davis *et al.*, 1988), distinguishes strains with chromosomal mutations (Chiou and Jones, 1995b). Although strains with low-level resistance have been identified in laboratory mutant searches, they are rare in nature, due to reduced fitness (Schroth *et al.*, 1979). It is possible that some strains of *E. amylovora* with chromosomal mutations for resistance may also contain genes that code for enzymes that modify streptomycin. Repeated attempts to detect *strA* and *strB* in HR strains of *E. amylovora* with point mutations were unsuccessful (McManus and Jones, 1994; Chiou and Jones, 1995b).

Mutations for high-level resistance

Mutation rates for streptomycin resistance were established in laboratory studies involving *E. amylovora* (Bennett and Billing, 1975; Schroth *et al.*, 1979). Spontaneous mutants with a high level of resistance to streptomycin were isolated at a frequency of 4.1×10^{-9} per bacterial generation and mutants with a low level of resistance were isolated at a frequency of 0.1×10^{-9} per bacterial generation. The mutants with low-level streptomycin resistance often failed to survive transfer to fresh media. These studies indicated that resistance to streptomycin in the field would probably originate from a single-step mutation and that the level of resistance would be high. Since all streptomycin-resistant *E. amylovora* collected from California pear orchards exhibited high-level resistance, it was concluded that streptomycin resistance arose in California through chromosomal mutation (Schroth *et al.*, 1979). Furthermore, no enzymes which modify streptomycin and no extra plasmids were found in these strains (Schroth *et al.*, 1979; Chiou and Jones, 1995b).

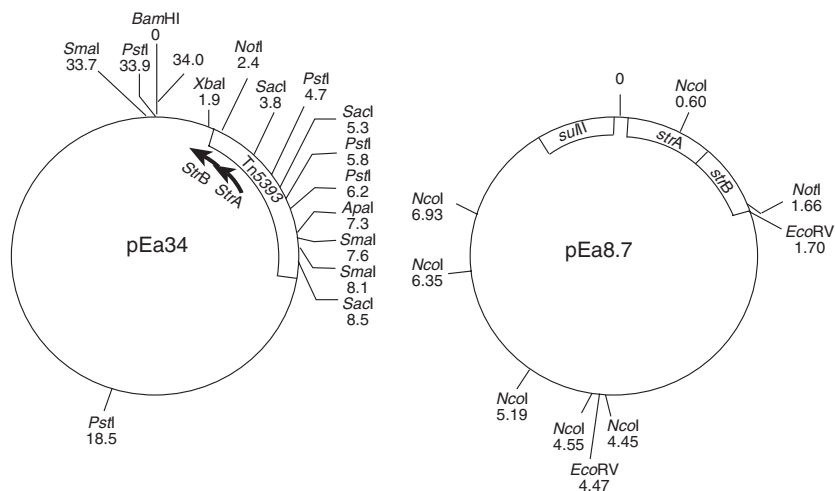
Streptomycin resistance can arise from mutations in the *rpsL* gene, which alter ribosomal protein S12; streptomycin cannot bind to the remodelled target site on the ribosome (Galili *et al.*, 1989). The *E. amylovora* *rpsL* gene is only 375 bp and therefore easily manipulated for genetic analysis. Using the nucleotide

sequence of the *rpsL* gene and allele-specific PCR analysis, Chiou and Jones (1995b) identified the mutations associated with streptomycin resistance in highly resistant strains of *E. amylovora* obtained from several states in the USA and from New Zealand. Each of the HR strains examined contained a mutation in the same codon of the *rpsL* gene. Codon 43, which encodes for lysine in sensitive strains, was converted to a codon for arginine in most HR strains or asparagine or threonine in a few others. These mutations had previously been reported to confer high-level resistance in *Escherichia coli* (Bohman *et al.*, 1984). Complementation analysis was used to prove that the mutations detected in the *rpsL* gene of field strains were responsible for high-level streptomycin resistance in *E. amylovora*. Resistant strains were changed to the streptomycin-sensitive phenotype by complementation with a high-copy-number plasmid carrying the wild-type *E. amylovora rpsL* gene; conversely, sensitive strains were changed to a resistance phenotype by complementation with a plasmid carrying the *rpsL* gene from HR strains (Chiou and Jones, 1995b). This study provided direct evidence that different alleles of the *rpsL* gene can cause high-level streptomycin resistance in *E. amylovora* and that one of these alleles is common in field strains.

Resistance genes, plasmids and transposons

Progress on the genetics of acquired resistance to streptomycin came quickly following the recognition that the resistance genes in Michigan strains of *E. amylovora* with a medium-level of streptomycin resistance (MR strains) were located on plasmid DNA (Chiou and Jones, 1991). Sequence analysis of the streptomycin resistance genes in *E. amylovora* strain CA11 revealed two linked genes, called *strA*–*strB*, which were originally identified in the broad-host-range plasmid RSF1010 found in a variety of clinical isolates of Gram-negative bacteria (Chiou and Jones, 1993). The genes *strA* and *strB* encode for aminoglycoside-3''-phosphotransferase (APH(3'')-Ib) and aminoglycoside-6-phosphotransferase (APH(6)-Id), respectively (Shaw *et al.*, 1993). These periplasmic enzymes confer resistance in *E. amylovora* through phosphorylation of the 3''- or 6-hydroxyl position of the streptomycin molecule, thereby rendering streptomycin impotent (Chiou and Jones, 1995a).

Further genetic analysis demonstrated that in *E. amylovora strA*–*strB* was part of a 6.7-kb transposable element designated transposon Tn5393, located on a 34-kb self-transmissible plasmid designated pEa34 (Fig. 12.1; Chiou and Jones, 1993). The transposability of this element was confirmed by showing that it could be copied from pEa34 and inserted into various sites on two other plasmids. The detection of Tn5393 on the non-conjugative plasmid pEA29 or integrated into the chromosome in some MR strains of *E. amylovora* from Michigan apple orchards also supports the transposability of this element (McManus and Jones, 1994). Tn5393 was sequenced and found to contain two 81-bp inverted repeats, one at each end, and divergently transcribed transposase (*tnpA*) and resolvase (*tnpR*) genes, separated by a transposon cointegrate



resolution site (*res*). An insertion sequence, *IS1133*, with a promoter sequence that increases the expression of the adjacent *strA-strB* genes, was found following the *tnpR* gene (Chiou and Jones, 1993, 1995a). Interestingly, a different insertion sequence (*IS6100*) in Tn 5393 was involved in the expression of *strA-strB* in *Xanthomonas campestris* pv. *vesicatoria* and no insertion sequences were involved in the expression of *strA-strB* in two pathovars of *Pseudomonas syringae* (Sundin and Bender, 1995).

Until 1994, streptomycin resistance associated with *strA-strB* had only been reported in *E. amylovora* from Michigan; analysis of streptomycin-resistant *E. amylovora* isolated from apple and pear orchards in other regions revealed a point mutation in the *rpsL* gene in each case. Palmer *et al.* (1997) found *strA-strB* associated with plasmid pEa8.7 in resistant strains isolated from an apple orchard in California. Unlike MR strains from Michigan, the MR strains from California were resistant to both streptomycin and sulphonamide antibiotics. Based on a combination of PCR, restriction and sequence analysis, it was established that *strA-strB* was genetically linked with the *suIII* sulphonamide-resistance gene on a plasmid closely related to RSF1010 (see Fig. 12.1) (Palmer *et al.*, 1997). Previously, RSF1010-like plasmids had been detected in a variety of clinically important bacteria but not in phytopathogenic bacteria (Sundin and Bender, 1996). Even though RSF1010-like plasmids are non-conjugative, in the presence of a plasmid encoding mobilizing functions they can be mobilized and spread to other bacteria. The detection of pEa8.7 in *E. amylovora*

indicates dissemination of this important broad-host-range plasmid to a new host and to a new ecological niche. Since the inverted repeat sequence of Tn 5393 is found in the flanking region of *strB* in RSF1010, it is interesting to speculate that the evolution of RSF1010-like plasmids and of Tn 5393 is somehow linked.

Epidemiology of streptomycin resistance

Although a number of factors can contribute to the development of populations of streptomycin-resistant *E. amylovora*, clearly streptomycin as the selective agent for resistance genes and new mutations is the most important. In the natural competition between susceptible and resistant bacteria, bacteria able to defy eradication by streptomycin will emerge and multiply, thereby shifting the balance in favour of resistant bacteria. Streptomycin is needed to select *E. amylovora* strains with random mutations for resistance, to select a reservoir of bacteria with transferable genes for resistance and to select *E. amylovora* strains that have acquired resistance genes from the resistance reservoir. Furthermore, continued use of streptomycin after the emergence of resistant genotypes allows evolving bacteria to compensate for possible deleterious side-effects associated with their recently acquired resistance genes.

Orchard reservoirs of *strA–strB* and the cycle of resistance

The detection of *strA–strB*, Tn 5393 and pEa 34 in streptomycin-resistant strains from Michigan and of *strA–strB* and pEa8.7 in resistant strains from California demonstrates that *E. amylovora* is evolving by acquiring new plasmids and genes. By knowing the source of this foreign DNA, it should be possible to develop strategies to break the cycle of acquired streptomycin resistance in *E. amylovora* (Fig. 12.2). Populations of both non-target and target bacteria are put under selection pressure to become streptomycin-resistant when streptomycin is used on apple and pear to control fire blight and other bacterial diseases. As a result, a reservoir or pool of Gram-negative bacteria that harbour *strA–strB* and Tn 5393 on plasmids is created (Norelli *et al.*, 1991; Sobiczewski *et al.*, 1991; Burr *et al.*, 1993; Vanneste and Voyle, 1996). Eventually, a microbe with *strA–strB* transfers a resistance plasmid by conjugation to a cell of *E. amylovora*, thereby creating an MR strain. The new strain is unlikely to be observed without selection pressure, but with streptomycin-mediated selection the resistant strain soon outnumbers the susceptible strains in the orchard population. Usage of streptomycin also favours the selection of bacteria with spontaneous mutations for resistance.

The Gram-negative, streptomycin-resistant bacteria commonly recovered from Michigan, New York and New Zealand apple orchards include an array of fluorescent pseudomonads and of non-fluorescent bacteria. *Erwinia herbicola*

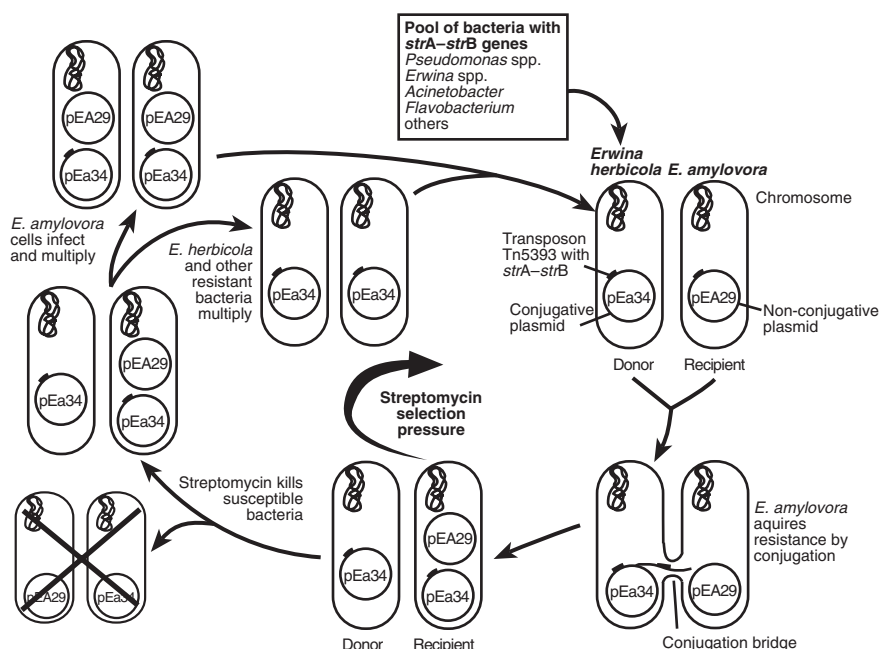


Fig. 12.2. The cycle of streptomycin resistance, showing a likely route by which *Erwinia amylovora* acquired the *strA-strB* genes and the role of streptomycin-mediated selection in the build-up of streptomycin-resistant strains. The resistance gene pool includes genera of streptomycin-resistant bacteria with *strA-strB* that are commonly found in apple orchards. Dissemination is brought about when *strA-strB* genes are transferred on a plasmid, such as pEa34 in one of the resistant bacteria, to *E. amylovora* by conjugation. Involvement of the transposon Tn5393 has been demonstrated for at least three genera of plant-pathogenic bacteria. The use of streptomycin for fire blight control reduces the population of streptomycin-sensitive bacteria, while favouring the build-up of streptomycin-resistant bacteria.

(*Pantoea agglomerans*) and fluorescent and non-fluorescent *Pseudomonas* species are commonly isolated from the surface of apple leaves and from fire blight-infected tissues (Norelli *et al.*, 1991; Sobiczewski *et al.*, 1991; Burr *et al.*, 1993; Vanneste and Voyle, 1996). In these bacteria it is common to find *strA-strB* on plasmids. The size of the streptomycin-resistance plasmids is variable among these bacterial epiphytes of apple and *strA-strB* genes are often detected on plasmids much larger than 34 kb (Huang and Burr, 1999). Although these bacteria contain a homologous gene for resistance, there is variation in the size of the *Ava*I restriction fragment that hybridizes with a *strA* DNA probe, due to polymorphism in Tn 5393. *Ava*I restriction fragments of 2.7, 1.5 and 0.9 kb are commonly detected by Southern analysis (Norelli *et al.*, 1991; Sobiczewski *et*

al., 1991). The difference between the 2.7- and 1.5-kb fragments is the presence or absence of IS1133 in Tn5393 (Chiou and Jones, 1993). The nature of the 0.9-kb fragment has not been investigated.

Among the many bacterial species that are reservoirs of *strA-strB* in Michigan apple orchards (Sobiczewski *et al.*, 1991), *E. amylovora* probably acquired plasmid pEa34 and the resistance transposon Tn5393 from *E. herbicola*. Insertion sequence IS1133 is associated with Tn5393 in all characterized MR strains of *E. amylovora* from Michigan; it is a highly discriminatory marker for finding possible donor species among the streptomycin-resistant bacterial epiphytes of apple. Some streptomycin-resistant strains of *E. herbicola*, a species ecologically associated with *E. amylovora*, harbour plasmid pEa34 and Tn5393 with IS1133 (Fig. 12.3). Neither IS1133 nor plasmid pEa34 has been associated with streptomycin-resistant pseudomonads from apple or other agricultural environments (Sobiczewski *et al.*, 1991; Chiou and Jones, 1993; Sundin and Bender, 1993). Experimentally, pEa34 was transferred at high frequency between *E. herbicola* and *E. amylovora* (Fig. 12.3; Chiou and Jones, 1993), as was one large resistance plasmid (Fig. 12.3), while attempts to transfer other *strA-strB*-containing plasmids into *E. amylovora* were unsuccessful (Huang and Burr, 1999). Although pEa34 has not been detected in *E. herbicola* outside Michigan (Norelli *et al.*, 1991; Burr *et al.*, 1993; Huang and Burr, 1999), our development of PCR primers for amplifying a fragment of pEa34 (see Fig. 12.3) should stimulate studies on the association of pEa34 with *E. herbicola* in other regions.

Interestingly, a 28-kb plasmid with homology to pEa34 but lacking transposon Tn5393 was found in a geographically isolated population of streptomycin-sensitive *E. amylovora* (Chiou and Jones, 1993). This observation indicates that this plasmid is compatible with *E. amylovora* even without streptomycin-resistance genes. Although *E. amylovora* may have acquired Tn5393, which was then inserted into the resident 28-kb plasmid, this scenario for the outbreak of resistance is unlikely, since the 28-kb plasmid has not been detected in sensitive strains of *E. amylovora* from orchards with MR strains. It is more likely that this 28-kb plasmid was acquired by *E. amylovora* from *E. herbicola*; it would be interesting to know whether this 28-kb plasmid is common in sensitive strains of *E. herbicola*.

Less is known about the source of the RSF1010-like plasmid pEa8.7. RSF1010 is non-conjugative but, being a broad-host-range plasmid, it can be mobilized and spread to other bacteria in the presence of a plasmid encoding mobilizing functions. Recently, an RSF1010-like plasmid was identified in *E. herbicola* from an apple orchard in New Zealand (J.L. Vanneste, personal communication). The wide distribution of RSF1010-like plasmids was reported previously only in clinical bacteria of animal and human origin (Sundin and Bender, 1996). The detection of RSF1010-like plasmids in *E. amylovora* in California and *E. herbicola* in New Zealand suggests that enteric bacteria have probably been intermingling outside the clinical realm, thus providing a way to disseminate this plasmid to new hosts.

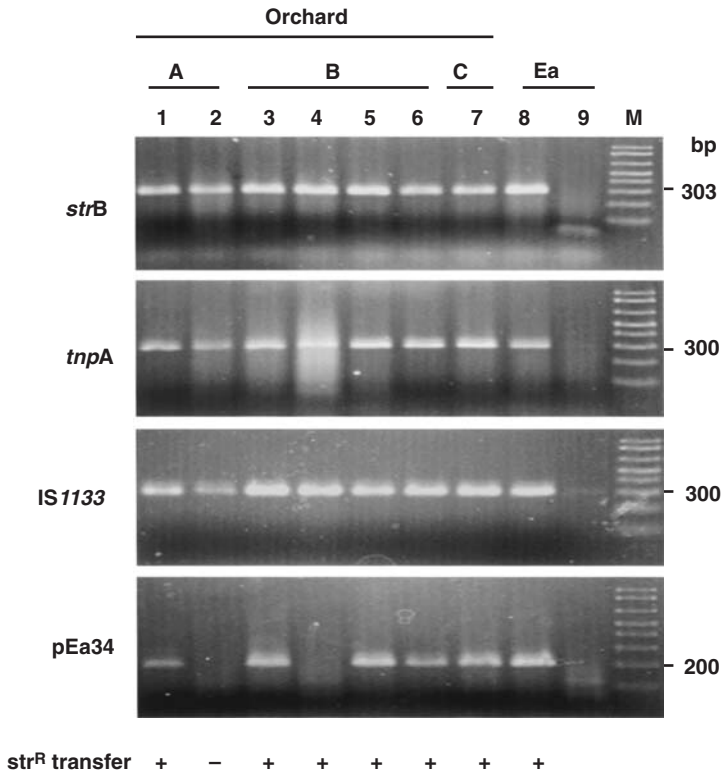


Fig. 12.3. Detection of *strB*, *tnpA*, IS1133 and plasmid pEa34 in a group of streptomycin-resistant *Erwinia herbicola* (lanes 1–7) isolated from three Michigan apple orchards. Lanes 8–9 are streptomycin-resistant *Erwinia amylovora* strain CA11 with Tn5393 (Chiou and Jones, 1993) and streptomycin-sensitive strain Ea110, respectively. The PCR primer pairs were *strB*-F GGAAGTGGCTGGGCTACA and *strB*-R GCTAGATCGCGTTGCTCCTCT for *strB*; *tnpA*-F CATTCCGAGACGCAAACA and *tnpA*-R CTATGTCGATCCGAGAACAGTT for *tnpA*; IS1133-F GGCGATATCACTTATGTTTGGA and IS1133-R GAAGCTTTCTACTGCGGAGTTA for IS1133; and pEa34-A AGTCGTGATTCCATTTTATTTTC and pEa34-B CGTTTATCGGATGCCTTTGAGTC for pEa34. The success (+) or failure (–) of conjugal transfer of plasmid DNA carrying streptomycin resistance to *E. amylovora* and to *Escherichia coli* is indicated along the bottom of the gel. The conjugative streptomycin-resistance plasmid from the isolate shown in lane 4 was larger than pEa34.

Tracing outbreaks of resistant strains

Streptomycin-resistant strains with medium resistance were detected in two widely separated counties in Michigan (McManus and Jones, 1995). Epidemiological studies to determine whether a single outbreak of resistance

was followed by migration of the resistant strain or whether resistance arose independently in each county and orchard have not been conclusive. Fruit-tree strains of *E. amylovora* were distinguished from *Rubus* strains by repetitive element PCR (rep-PCR) and by PCR ribotyping, but sensitive strains and MR strains from tree fruit crops were genetically homogeneous and therefore indistinguishable (McManus and Jones, 1995). Plasmid pEA29 is unique to *E. amylovora* and PCR amplification from pEA29 is a common assay for the detection and identification of *E. amylovora* (Bereswill *et al.*, 1992). The PCR-amplified fragment of pEA29 contains a region of 8-bp repeats, which might be useful for differentiating strains of *E. amylovora*. However, due to instability in the number of repeats, this method was not useful for typing streptomycin-resistant and sensitive strains of *E. amylovora* (Schnabel and Jones, 1998). Recently, an analysis of MR strains indicated that Tn5393 was inserted at the same location in pEa34 of all strains of *E. amylovora* but in at least two locations in strains of *E. herbicola* (E.L. Schnabel and A.L. Jones, unpublished observation). These preliminary data suggest that the outbreak of acquired resistance in Michigan may have arisen from a single or a few rare transfers of pEa34 to *E. amylovora*, followed by migration.

The initial outbreak in Michigan of MR strains of *E. amylovora* with self-transmissible plasmid pEa34 was followed by outbreaks of MR strains associated with plasmid pEA29::Tn5393, a plasmid formed when non-transmissible pEA29-acquired Tn5393 (McManus and Jones, 1994). MR strains with Tn5393 inserted on the chromosome were also found, but in very low frequency. In 1999, an outbreak of resistant strains of *E. amylovora* on three adjacent farms was associated with plasmid pEA29::Tn5393 (E.L. Schnabel and A.L. Jones, unpublished observation). Differentiating strains with pEA29::Tn5393 from those with pEa34 can be accomplished by PCR amplification of sequences flanking the *strB* gene, followed by sequence analysis of the amplified fragment.

Stability of streptomycin resistance

The effect of plasmids and mutations for resistance on the fitness of *E. amylovora* in the absence of streptomycin-mediated selection will have an impact on the persistence of resistant strains in orchards and the extent to which streptomycin can ever be reintroduced following a resistance episode. In California, Washington and Oregon, strains of *E. amylovora* with a high level of resistance to streptomycin were detected many years after the use of streptomycin was discontinued (Schroth *et al.*, 1979; Loper *et al.*, 1991; Stockwell *et al.*, 1996). This observation indicated that the long-term survival of HR strains had not been affected adversely by the mutations for resistance. In addition, there was no difference in generation times and virulence between streptomycin-sensitive and streptomycin-resistant strains of *E. amylovora*, indicating that fitness had not been impaired by resistance mutations in HR strains (Schroth *et al.*, 1979).

Mutations for high-level resistance to streptomycin in *E. amylovora* have probably evolved independently at least twice. HR strains from the western USA were found to have a different PCR ribotype from that of HR strains from Michigan and New Zealand (McManus and Jones, 1995). The evolution of resistance to streptomycin in HR strains of *E. amylovora* from the USA and New Zealand has favoured base-pair mutation in codon 43 of the *rpsL* gene, which results in the substitution of arginine for lysine in ribosomal protein S12 (Chiou and Jones, 1995b). In *E. coli*, a lysine-to-arginine mutation at this location had a negative effect on fitness as measured by decreasing peptide chain elongation rates, although the effect was less dramatic than lysine-to-threonine or lysine-to-asparagine mutations (Bohman *et al.*, 1984). However, following the initial period of reduced fitness, beneficial mutations can soon restore fitness without altering the level of resistance (Schrag and Perrot, 1996). Thus, it is likely that, by the time a streptomycin-resistant population can be detected in the field, fitness has been restored and the resistant bacteria can survive in the absence of continued streptomycin-mediated selection.

It appears that the carriage of resistance plasmid pEa34 has very little impact on the fitness of bacteria. Strains of *E. amylovora* with pEa34 were detected in a Michigan apple orchard 7 years after resistance was first detected and streptomycin application halted, suggesting that resistant strains will persist even where streptomycin has been replaced by oxytetracycline and copper (A.L. Jones, unpublished data). There was no difference in the amount of blossom blight produced by two strains of *E. amylovora* that were genetically identical except for the presence or absence of pEa34 (McManus and Jones, 1994). Similarly, streptomycin-resistance plasmids with Tn5393 did not alter the fitness of *P. syringae* pv. *syringae* (Sundin and Bender, 1994). These observations are consistent with theoretical studies demonstrating the ability of bacteria to adapt to their resistance (Lenski, 1997). Since pEA29 is a non-transferable plasmid that is unique to *E. amylovora*, the evolution of plasmid pEA29::Tn5393 has fixed the *strA-strB* genes in *E. amylovora*. Resistance associated with pEA29::Tn5393 will be inherited by daughter cells, but resistance transfer to susceptible strains will be unlikely.

Resistance management strategies

Management plans are needed for preventing resistance in areas where it has not developed, for controlling blight when resistance develops and for reintroducing streptomycin if resistant populations decline to manageable levels. Management plans are also needed to reduce the risk of dispersal from orchards with resistance. In areas where resistance problems have not emerged, increased emphasis on preventing resistance through more judicious use of streptomycin, combined with careful cultivar and rootstock selection, aimed at avoiding highly susceptible types, and orchard sanitation, aimed at preventing disease build-up, will be important management strategies. Fire blight control

will be very difficult in areas where streptomycin-resistant strains already exist. In these cases, increased emphasis on the development and use of alternative antimicrobial agents will be particularly important.

Reduction in the use of streptomycin

The question of why *E. amylovora* developed resistance to streptomycin many years earlier in the western USA than in the eastern USA was raised following the development of resistance in California (Moller *et al.*, 1981). The answer probably relates to marked differences in the level of streptomycin-mediated selection between the two regions. After streptomycin was introduced for fire blight control in the 1950s, the amount of streptomycin used by pear growers in western states was much greater than the amount used by apple growers in eastern states. In the western USA, streptomycin was applied at regular intervals for a minimum of 10–14 applications per season (Moller *et al.*, 1981). Although most of the streptomycin applications were applied during bloom, the regimen also included pre-bloom and post-bloom applications no later than 90 days before harvest. It was not until the early 1970s, when streptomycin resistance was emerging, that pre-bloom applications were shown to be unnecessary (Thomson *et al.*, 1975). However, the benefits of reduced selection pressure due to the elimination of pre-bloom applications were offset in part because streptomycin was then being used up to 30 days before harvest.

In the eastern USA, streptomycin was applied up to five times per season, with most growers applying two to three applications focused during bloom. Initially, the timing of bloom sprays was based on growth stage only, but by the early 1960s applications of streptomycin were being withheld until environmental conditions were judged favourable for infection, based on criteria developed and promoted by Mills and Parker (Luepschen *et al.*, 1961) and later by Steiner and Lightner (1992). Because streptomycin was not applied until conditions became favourable for infection, the selection pressure for resistance from multiple applications year after year as practised in western states was largely avoided in eastern states. However, growers in Missouri were encouraged to routinely apply pre-bloom applications of streptomycin in addition to bloom sprays (Goodman, 1982). This practice increased the streptomycin selection pressure and was followed by problems with streptomycin resistance (Shaffer and Goodman, 1985). In Michigan, resistance developed primarily in orchards where streptomycin was included at low rates in post-bloom pesticide applications up to 50 days before harvest or until cessation of terminal growth (A.L. Jones, unpublished data). Although streptomycin-resistant *E. amylovora* probably arose several times in both regions, either by chromosomal mutation or by gene acquisition, the historical evidence indicates that streptomycin-mediated selection pressure in the eastern USA was not sufficient to increase resistant populations to detectable levels, as occurred in the western USA.

Combining streptomycin with oxytetracycline

When streptomycin was initially introduced in the 1950s, it was sometimes used in combination with oxytetracycline to avoid possible resistance problems (Moller *et al.*, 1981). This strategy was abandoned because streptomycin alone was effective and cheaper. Also, the amount of oxytetracycline in the combination was probably too low to provide adequate resistance management. A mixture of two antibiotics should favour the predominance of antibiotic-susceptible bacteria, provided that resistance to each antibiotic arises independently. In laboratory studies, streptomycin-resistant strains of *E. amylovora* were selected in fewer generations in media containing streptomycin alone than in media containing both antibiotics (English and Van Halsema, 1954). Today, in areas of the USA where streptomycin resistance is emerging, pear and some apple growers are using combinations of streptomycin and oxytetracycline, with each antibiotic at full rate, for fire blight control.

More research is needed on the value of antibiotic mixtures for improving overall fire blight control and delaying the development of resistance to streptomycin and oxytetracycline in *E. amylovora*. Recent attempts to detect tetracycline-resistant *E. amylovora* were negative (Loper *et al.*, 1991; Stockwell *et al.*, 1996). Laboratory studies indicate that two or more mutational steps are required for *E. amylovora* to develop tetracycline-resistant strains with a level of resistance sufficient to interfere with disease control (Lacy *et al.*, 1984). Chromosomal mutants with tetracycline resistance would therefore be expected to arise in nature much less frequently than mutants with streptomycin resistance. In clinical bacteria, tetracycline resistance usually arises through gene acquisition (Roberts, 1996). One of the resistance genes commonly acquired by Gram-negative bacteria, *tetA*, has been shown to function in *E. amylovora* (Cho *et al.*, 1975; Lacy *et al.*, 1984). Among tetracycline-resistant bacterial epiphytes isolated from Michigan apple orchards, five related tetracycline-resistance genes – *tetA*, *tetB*, *tetC* and two variants of *tetG* – were detected (Schnabel and Jones, 1999). The tetracycline-resistance genes were almost always found on large plasmids, which also carried the *strA*–*strB* gene pair in Tn 5 393; however, these large plasmids failed to grow in *E. amylovora* (E.L. Schnabel and A.L. Jones, unpublished data, 1999). Therefore, combining streptomycin with oxytetracycline for fire blight control would be likely to result in the selection of a pool of bacteria resistant to both antibiotics. However, it has been suggested that the conjugal transfer of most plasmids conferring streptomycin-resistance to *E. amylovora* would occur rarely (Huang and Burr, 1999). Furthermore, when plasmids carrying both tetracycline- and streptomycin-resistance genes were introduced into *E. coli*, the streptomycin phenotype was weakly expressed (Schnabel and Jones, 1999). Therefore, even if transfer did occur, it has not been established that the resistance to streptomycin would be expressed in *E. amylovora*.

Summary

The introduction of streptomycin for fire blight control in the 1950s was eventually followed by the evolution of streptomycin resistance in *Erwinia amylovora*. The resistance phenomenon was first detected in California in the early 1970s and then in apple and pear orchards throughout most of the western USA. In this region, streptomycin resistance was due to the appearance of point mutations in the ribosomal protein S12 gene that alter the streptomycin target site. Investigations of streptomycin resistance in *E. amylovora* in Michigan in the early 1990s generated some new insights into how plant-pathogenic bacteria transfer resistance genes. A pair of transmissible streptomycin-modifying genes (*strA*–*strB*), located in transposon Tn 5393 on plasmid pEa34 and later on plasmid pEA29, were identified as the cause for the outbreak of resistant *E. amylovora*. These genetic elements, except pEA29, were also found in several Gram-negative bacteria associated with apple. These bacteria and particularly *E. herbicola* were the likely reservoir for the streptomycin resistance acquired by *E. amylovora*. Subsequently, acquired resistance was detected in *E. amylovora* from one orchard in California where *strA*–*strB* genes were associated with an 8.7-kb RSF1010-like plasmid rather than the 34-kb plasmid found in Michigan. Today, streptomycin resistance is fixed in *E. amylovora* in several apple- and pear-growing regions and more effective alternative strategies, both chemical and non-chemical, need to be developed for fire blight control.

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Breeding for Resistance to Fire Blight

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Introduction

Nearly all cultivars of economically important fruits and ornamental plants that belong to the *Maloideae* are propagated by cloning. Furthermore, given favourable environmental conditions, cultural practices may increase the severity of fire blight to destructive levels. A commercial planting of fruit trees usually consists of one cultivar and its pollinizer, which can also result in an increased level of damage if the cultivar is susceptible to fire blight. A second important factor explaining the destructive effect of fire blight is the long life of an orchard, which allows the build-up of high populations of the bacterium.

No commercial crop receives more chemical pesticides than fruits. This is due, in addition to the cultural factors mentioned above, to the complete range of plant parts which may be attacked and to the long period during the growing season when the pathogens may be active. High plant densities, while improving some aspects of production efficiency, can increase the incidence of fire blight. No chemical can completely control the bacterium (Psallidas and Tsiantos, Chapter 11), and the use of antibiotics, where permitted, has resulted in the development of resistant strains (Jones and Schnabel, Chapter 12).

The need for highly disease-resistant cultivars is more pressing than ever. Genetic disease immunity or resistance is recognized as an important feature of integrated pest management (IPM). Breeding disease-resistant fruit and ornamental tree cultivars usually involves combining disease resistance with the best characters of susceptible cultivars. Horizontal resistance, which implies the possible development of at least some infection, might be useful as long as crop loss is held to an acceptably low level – as in the case of fire blight. Genetic resistances are more often identified in primitive species or forms, or in obsolete

cultivars with mediocre appearance and quality. The several generations required to combine the best characteristics of one cultivar with disease resistance explain the necessity for long-term and carefully planned breeding programmes. The long generation time in fruit and ornamental trees may require continued breeding efforts through several decades. The pomologist, if working alone as a disease-resistance breeder, must therefore be highly knowledgeable about the disease in question. Preferably, he/she should work in close cooperation with a plant pathologist. Even better is an integrated team approach, involving efforts of scientists in both disciplines working in two or more areas representing somewhat different sets of environmental conditions. Team efforts have the greatest potential for rapid progress toward planned objectives.

Evaluation of species and cultivar susceptibility

Fruit scion and rootstock cultivars

Natural infection in orchard or nursery

Traditionally, resistance to fire blight has been evaluated by observing seedlings or trees growing under nursery or orchard conditions. Several factors other than plant genotype can affect these ratings, since natural sources of inoculum, cultural practices and environmental conditions vary from orchard to orchard, and even within orchards (Aldwinckle and Beer, 1978). Rootstocks (Keil and van der Zwet, 1975), tree age (Shaw, 1934), orchard topography and soil type (Fisher *et al.*, 1959) and inoculum level (Beer, 1978) affect the disease and thus may obscure inherent genotypic differences in susceptibility.

Nevertheless, reports based on field observations provide an important check on the validity of more artificial but better controlled experiments and, for some cultivars, are the only information available. The most comprehensive reports about apple susceptibility are those of Thompson (1972) and Aldwinckle *et al.* (1976), and about pear susceptibility the report of Oitto *et al.* (1970). Scores used by van der Zwet *et al.* (1970) ranked from 0 to 10, with the higher scores (10–8) indicating the least damage. This system is an overall appraisal which does not take account of the origin of the infection, usually flowers or shoots. In fact, it was demonstrated (van der Zwet, 1975) that susceptibility could vary widely depending on the organs affected. Studying a large number of pear selections and cultivars in different locations (Maryland and Ohio), van der Zwet *et al.* (1984) showed inconsistencies in the comparative results, especially in the moderately resistant and resistant classes and occasionally even among the fairly susceptible classes.

Van der Zwet and Keil (1979) published ratings of cultivars for fire blight resistance based on the literature; these ratings covered European and Asian pear, and apple cultivars. They also reported an overall degree of fire blight resistance for different *Pyrus* species. The cultivars were grouped into four classes: highly resistant, resistant, moderately resistant and susceptible.

PEAR CULTIVARS (*Pyrus communis*). Of the 287 cultivars named prior to 1920, only 11% are resistant to highly resistant; of the 113 cultivars released between 1920 and 1978, about one-third were reported to be predominantly resistant. As sources of resistance, van der Zwet and Keil (1979) ranked, in descending order based on their degree of fire blight resistance, *Pyrus ussuriensis*, *Pyrus calleryana*, *Pyrus betulaefolia*, *Pyrus pyrifolia* and *Pyrus communis*. Of the 49 clones of sand pear (*P. pyrifolia* synonymous with *Pyrus serotina*), only 28% were in the resistant classes.

PEAR ROOTSTOCKS. Quince, which is largely used as a pear rootstock, is usually susceptible to fire blight. A new series of rootstocks are being developed from pear, mainly from the cultivar 'Old Home', which is considered resistant to fire blight (Lombard and Westwood, 1987).

APPLE CULTIVARS (*Malus × domestica*). Of the 193 cultivars introduced before 1920, about 28% are resistant; of the 197 cultivars released between 1920 and 1978, 41% are resistant.

APPLE ROOTSTOCKS. Several clonal apple rootstocks are highly susceptible to *Erwinia amylovora* (Cummins and Aldwinckle, 1973). Of the 28 clonal rootstocks (Malling and Malling–Merton series), only nine were in the light susceptibility class. The most important rootstock, M.9, is in the severe susceptibility class.

INTERACTION BETWEEN SCION AND ROOTSTOCK CULTIVARS. Rootstock influence on the growth habit of the scion may also affect scion susceptibility to fire blight. For example, a later flowering period, when conditions are more favourable for infection by *E. amylovora*, can lead to an increase in fire blight incidence.

Artificial inoculation

To avoid the effect of the large variations in inoculum levels that prevail under natural conditions, artificial inoculation techniques have been developed for selection in breeding programmes. The most useful index for fire blight susceptibility is the extent of lesion development on the shoot. Measurements of this type have been shown to be strongly correlated with the field susceptibility of apple cultivars in several independent observations. Quamme *et al.* (1976) have also provided evidence that determination of fire blight susceptibility of pear cultivars by artificial inoculation is a valid procedure.

Blossom susceptibility of apple and pear cultivars has received less attention than susceptibility of vegetative tissues. However, susceptibility to blossom infection may be important in determining how readily infections are initiated in the orchard (Aldwinckle and Norelli, 1981). Correlation between susceptibility of shoots and of flowers is weak ($0.25 < r < 0.44$) (Thibault and Le Lezec, 1990); a good illustration is the cultivar 'Gala', which is only slightly susceptible on shoots but highly susceptible on flowers.

In orchard or nursery plantings, artificial inoculation of either shoot tips or blossoms may be more or less successful depending on weather conditions. However, if trees under similar growing conditions are inoculated identically and simultaneously, they can be compared in terms of susceptibility.

Several pome-fruit breeding programmes currently evaluate progeny for susceptibility to fire blight in the greenhouse. These methods appear to be the most efficient in eliminating highly susceptible individuals and are a great improvement over observations of natural infections in the field (Aldwinckle and Preczewski, 1976; Quamme, 1977; Aldwinckle and van der Zwet, 1979).

Aldwinckle and Preczewski (1976) artificially inoculated 92 apple cultivars; results indicate that, in contrast to pears, some important apple cultivars do have considerable resistance to fire blight – for example, ‘Delicious’ and its sports, and ‘Winesap’. Some of the scab-resistant cultivars are also resistant to fire blight, such as ‘Priscilla’ and ‘Liberty’; fire blight-resistance genes were probably transmitted fortuitously along with scab-resistance genes from *Malus floribunda* 821, the source of the *Vf* gene. Moreover, Aldwinckle and Preczewski (1976) found that two spur-type apple cultivars were significantly less susceptible to fire blight than their standard-type parent cultivars.

In most artificial inoculation experiments, a certain number of twigs do not react after inoculation. At the Institut National de la Recherche Agronomique (INRA) Angers laboratories, it was decided to take into account both the frequency (F, 0–1) and the severity (S, 0–100) of infection; this led to the index of varietal susceptibility (IVS) (Thibault *et al.*, 1987):

$$\text{IVS} = F \times S, (0-100)$$

The IVS is divided into five classes (I to V) from 0 to 100 (from resistance to high susceptibility). This index is more reliable with data from several years; the evaluation performed at INRA Angers associated incidence (classes 1 to 5), severity (classes 1 to 5) and IVS (0–100) to characterize a cultivar. Seventy-six apple cultivars and 85 pear cultivars were assessed in this way (Le Lezec *et al.*, 1985; Thibault and Le Lezec, 1990). A complete report of these studies was recently published (Le Lezec *et al.*, 1997).

In this review, we present ratings of the most important apple and pear cultivars (scions and rootstocks) currently grown in the world. These results were obtained in France over 3 years after artificial inoculation of the shoots in the field with *E. amylovora* (strain CFBP 1430) using a syringe; 36 shoots were inoculated per cultivar and per year.

Major apple cultivars are shown in Table 13.1. The group originating from the cultivar ‘Delicious’ is ranked highest for resistance (I = 1, S = 2, IVS = 11); more recent commercial cultivars, such as ‘Gala’, ‘Jonagold’, ‘Fuji’ and ‘Elstar’ are in the same class as ‘Golden Delicious’. Major pear cultivars are shown in Table 13.2. Among rootstocks (Table 13.3), the quince selections are moderately susceptible (class III). The new pear selections show good results (classes I and II); all share the same parent ‘Old Home’. Most commonly used apple rootstocks in Europe (M.9, M.26, MM.106) were susceptible or very susceptible in

Table 13.1. Ratings of the main apple cultivars grown in the world (from Le Lezec *et al.*, 1997).

Classes	Cultivar	Incidence (1–5)	Severity (1–5)	Index (IVS)
I 0–20	‘Delicious Spur’ ^a (‘Starkrimson’)	1	2	11
	‘Belle de Boskoop’	1	2	12
	‘Delicious Standard’ ^a (‘Hi-Early’)	1	3	15
	‘Golden Spur’ ^a	1	3	15
II 20–40	‘Jonagold’	2	2	21
	‘Gala’	3	3	24
	‘Reinette Grise du Canada’	4	2	27
	‘Golden Delicious’ ^a	3	3	33
	‘Elstar’	3	4	34
	‘Fuji’	5	2	36
III 40–60	‘Granny Smith’	5	3	45
	‘Braeburn’	5	3	52
	‘Melrose’	5	3	54
IV 60–80	‘Rome Beauty’	5	4	60
	‘Cox’s Orange Pippin’	5	4	68
	‘Idared’	5	4	68
	‘Reine des Reinettes’	5	5	71

^a The spur types are less susceptible than the standards (‘Delicious’ and ‘Golden’ types).

this trial. In the USA, the natural incidence of fire blight infection has been most severe on M.9 and M.26, with few problems on MM.106. There is a great need for a fire blight-resistant rootstock that confers similar size control to that of M.9.

There are other reports of the susceptibility of apple and pear (Zeller, 1978, 1990; Maroofi and Mostafavi, 1996). Twenty-four cultivars of Asian pears were evaluated in the field after inoculation; most of the ‘Nashi’ cultivars (*P. pyrifolia*) are susceptible; the ‘Li’ cultivars (*P. ussuriensis* and *Pyrus bretschneideri*) appeared less susceptible than the ‘Nashi’ cultivars (Lecomte, 1993). Most of the cider apples grown in the south-west of England (Gwynne, 1984) and in the west of France (Paulin *et al.*, 1990, 1993b; Chartier *et al.*, 1992) are susceptible to fire blight; the results obtained in France showed that shoot and blossom susceptibility is more extreme than in dessert apples. Crab-apple cultivars have also been evaluated (Cline, 1985; Bonn and Elfving, 1990); some fire blight-resistant crab-apples may be suitable both for apple orchards as pollinizers and for the ornamental industry.

Table 13.2. Ratings of the main pear cultivars grown in the world (from Le Lezec, *et al.*, 1997).

Classes	Cultivar	Incidence (1–5)	Severity (1–5)	Index (IVS)
I 0–20	‘Harrow Sweet’	2	1	11
	‘Conference’	3	1	16
	‘Beurré Bosc’	2	3	18
	‘Blanquilla’	4	1	18
	‘Coscia’	2	2	18
II 20–40	‘Docteur Jules Guyot’	4	2	21
	‘Beurré Hardy’	2	3	25
	‘Beurré d’Anjou’	2	5	30
	‘Rocha’	4	3	39
III 40–60	‘Abbé Fetel’	3	4	43
	‘Bartlett’	4	4	57
IV 60–80	‘Passe Crassane’ ^a	4	5	71
	‘Packham’s Triumph’	5	4	72
V 80–100	‘Doyenné du Comice’	5	5	90

^a Very susceptible on secondary blossom.

Ornamentals

Cotoneaster, *Pyracantha* and *Crataegus* are the most important genera of ornamentals susceptible to fire blight. Several studies on fire blight susceptibility of species or cultivars of *Cotoneaster* have been carried out in northern Europe (Zeller, 1979; Bouma, 1990a; Cadic and Lecomte, 1990). Results of about 55 cultivars of *Cotoneaster* have been summarized by Lecomte and Cadic (1993); most cultivars were susceptible.

Limited experimental data are available on seedlings of *Crataegus* species (Maas Geesteranus and Heyting, 1978; Bouma, 1990a). Paulin *et al.* (1993a) assessed 84 *Crataegus* species after shoot inoculation in the field; results showed that susceptibility varied widely within this genus – cases of extreme susceptibility were observed, as well as cases of apparently complete resistance. Paulin and Cadic (1997) listed some interesting resistant clones that are commercially useful ornamentals.

Bouma (1990a) and Cadic and Lecomte (1990) reported fire blight susceptibility of several species and cultivars of *Pyracantha*. This genus appeared less susceptible than *Cotoneaster*. Some cultivars of *Pyracantha atalantioides*, including ‘Gibbsii’ and ‘Debussy’, are very susceptible and have disappeared over time; some others, including ‘Shawnee’ and ‘Mozart’, are quite resistant.

Table 13.3. Ratings of the main rootstocks for pear and apple grown in the world (from Huet and Michelesi, 1990; Le Lezec, *et al.*, 1997).

Classes	Cultivar	Incidence (1–5)	Severity (1–5)	Index (IVS)
I <i>Pyrus</i> 0–20	INRA® ‘Pyriam’ (OH11)	1	2	11
	Delbard® 333 ‘Brokmal’	1	2	13
	Farold® 87 ‘Daytor’	3	2	14
	Farold® 282 ‘Dayre’	3	2	15
	Farold® 40 ‘Daygon’	4	2	18
	Delbard® 51 ‘Broklyl’	2	2	20
II <i>Pyrus</i> 20–40	Farold® 69 ‘Daynir’	3	2	26
III <i>Cydonia</i> 40–60	BA 29	5	3	53
	C.EM	5	3	55
	A.EM	4	2	58
	‘Sydo’	5	3	58
	‘Adam’s’	5	3	58
I <i>Malus</i> 0–20	‘Novole’	1	1	10
	MM.104	1	1	10
	‘Robusta 5’	1	1	11
	M.2	3	2	15
	MM.109	2	3	15
	MM.111	1	3	17
II <i>Malus</i> 20–40	M.7	4	2	24
	M.25	3	3	28
III <i>Malus</i> 40–60	M.27	5	3	50
	‘Pajam 2’ (M.9)	5	3	51
	‘Pajam 1’ (M.9)	5	4	60
IV <i>Malus</i> 60–80	‘Supporter 4’ (PI80)	5	4	69
	M.26	5	4	73
	MM.106	5	4	79

Breeding programmes

Van der Zwet and Keil (1979) reviewed breeding programmes on pear, apple, *Pyracantha*, hawthorn and *Cotoneaster*. This review emphasizes the breeding work from the late 1970s to the present.

Development of inoculum

Greenhouse screening requires the identification, isolation, maintenance and testing of the pathogenicity of the inoculum. Tests should include isolates from

diverse geographical areas and from plants from the same or closely related species and genera. The breeder must determine as quickly as possible the level of resistance in potential parents against isolates selected for their pathogenicity. Plant pathologists have developed a wide range of techniques for successful preservation of virulent inoculum and for the inoculation of host tissue.

Differences in virulence (amount of disease caused by a pathogen) are common among strains of *E. amylovora*. Unless there is an interaction between one or several cultivars tested and the strain, a strain is expected to have the same level of virulence, whatever the inoculated cultivar.

Specific interactions, called differential virulence, have been found between some strains of *E. amylovora* and some apple cultivars by Norelli *et al.* (1984, 1986, 1987). Strains with differential virulence to apple are not rare in North America (about 10% of the strains tested by Norelli, 1986), but they have never been found in Europe (J.P. Paulin, France, 1998, personal communication). In contrast, no evidence for such interaction has been found in *Pyrus* (Quamme and Bonn, 1981). In another study with pear, Bell and van der Zwet (1996), found no consistent isolate \times host genotype interaction, suggesting that differential virulence is not a major concern in breeding for fire blight resistance in pear using the current sources of resistance.

The use of a mixture of different strains, as recommended by Norelli (1986), can offer a solution to this problem. Paulin and Lespinasse (1990) suggested the inoculation of several selected strains in successive independent steps, since the use of a mixture of strains did not give a higher overall incidence and severity than the most virulent strain. Bell *et al.* (1990) studied 11 genotypes of pear (*P. communis*) and five strains of *E. amylovora* in various combinations at three locations for 2 years in order to assess the degree of variability in disease expression due to random strain and environmental effects. None of the interactions resulted in significant differences. Therefore, the choice of standard resistant and susceptible hosts can be arbitrary. Standard strains should, of course, be chosen on the basis of a high general virulence and frequency of successful infection.

Pear scion breeding programmes

Plants derived from *P. ussuriensis* and *P. serotina* led to several cultivars, including 'Magness', 'Lee', 'Mac' and 'Star', which were a compromise between resistance and fruit quality. Certain cultivars (e.g. 'Magness') showed an unexpected susceptibility to trunk blight. *P. ussuriensis* and *P. serotina* do not appear to be sources of resistance superior to that of the European pear, *P. communis* (Layne, 1964; Bell *et al.*, 1996).

The pear breeding programme conducted at the US Department of Agriculture (USDA) Appalachian Fruit Research Station, Kearneysville, West Virginia

This programme has generated many selections resistant to fire blight. At present, eight pear clones from a collection of 200 second-test selections have been proposed for further trials. All have shown resistance to infection equal to or better than that of 'Seckel', from which their resistance is derived (Bell and van der Zwet, 1993).

Studying some factors affecting selection for fire blight resistance in pear, van der Zwet *et al.* (1981) indicated that the presence of bloom and the transition to the adult phase are not necessarily a major factor in susceptibility to fire blight. They also supported early screening of juvenile seedlings.

The Agriculture and Agri-Food Canada pear breeding programme at Harrow, Ontario

Many sources of resistance have been used, including cultivars and selections from *P. communis*, *P. ussuriensis* and *P. pyrifolia*, with the fruit characteristics of *P. communis* being recovered by back-crossing to selected *P. communis* cultivars. Actively growing seedlings 30–40 cm tall grown in the greenhouse were inoculated near the shoot tip with a standardized suspension of six strains of *E. amylovora*. Upon evaluation 2 months later, seedlings were discarded if the inoculated lesion extended beyond 25–30% of the shoot length. The more resistant seedlings were planted out in the field, where the incidence and severity of natural fire blight infections were rated annually, using the USDA scale (van der Zwet *et al.*, 1970). Seedlings were screened again for fire blight resistance when they began to fruit, usually 5–7 years after establishing the seedling orchard, using the same technique as in the greenhouse (Hunter, 1993). 'Harrow Delight' and 'Harvest Queen' were named and released in 1981 (Quamme and Spearman, 1983) and 'Harrow Sweet' in 1990 (Hunter *et al.*, 1992).

Quamme *et al.* (1990) tested the efficacy of early selection for fire blight resistance. A set of progenies was tested for fire blight resistance by needle inoculation at 3 months of age in the greenhouse and then 5 years later in the orchard. Correlation of fire blight resistance at the two stages of growth was weak or absent on a single-plant basis. Nevertheless, genetic gain based on the field measurements appeared to be possible if plants were selected in the greenhouse with less than 20% of shoot length blighted. It was proposed that selection for fire blight be carried out at both stages of growth – in the greenhouse, as simple measurements on young plants, and again in the field, when replicated measurements can be made.

The INRA pear breeding programme at Angers, France

Thibault (1981) developed an initial programme for fire blight resistance breeding of pear from a half-diallel, including seven parents: four resistant American

selections and three old European cultivars. In contrast to a diallel cross, which is the set of all possible matings between several genotypes (varieties or selected clones), a half-diallel in pear does not include the reciprocal crosses and the combinations resulting from self-fertilization (the pear is self-sterile). Twenty-one progenies were obtained and 4000 hybrids were studied (Thibault and Maas Geesteranus, 1984). Later on, the programme was limited to *P. communis* parents with only low or moderate susceptibility. There were 55 parents and 200 progenies with a total of 55,000 hybrid seedlings (Thibault, 1990). Today 10,000 seedlings from this programme are under evaluation in the field for pomological traits, such as fruit quality (Le Lezec *et al.*, 1991). Thibault *et al.* (1987) compared two methods of selection of young seedlings: selection in the greenhouse on 30–40-cm-tall plants and selection in the field during the juvenile phase. Clearly, it appears that the selection in the greenhouse was too severe. The pear seedlings in the greenhouse are now selected for resistance if less than 50% of shoot length is blighted after inoculation with a single strain (CFBP 1430). This strategy permits maintaining enough plants per cross in the field to be able to evaluate the genetic interest of the parents and to increase the efficiency of the selection procedure for releasing new cultivars with desirable pomological traits. The other major character selected against is secondary blossom, which is only visible in the field at the adult stage. This trait is heritable (Thibault *et al.*, 1983). The strategy developed emphasizes the absence of secondary blossoms and lowers the percentage of shoot length blighted. The most recent pear cultivar released, 'Angelys' (1998), is a good example of this strategy; the future development of this new cultivar will be a good test of the paradigm.

The pear breeding programme conducted at the Istituto Sperimentale per la Frutticoltura, Rome and Forli, Italy

The programme emphasizes the breeding of dwarf and semi-dwarf cultivars suitable for high density orchards to be managed from the ground (Fideghelli *et al.*, 1984; Quarta, 1990). The population studied consisted of 107 progenies. The seedlings obtained were inoculated in the nursery, where conditions varied from year to year; the same progeny inoculated partly one year and partly another year could give quite different results in percentage of selected seedlings. In these conditions, standard cultivars, such as 'Stark Delicious', 'Bella di Giugno', 'Coscia' and 'Max Red Bartlett', susceptible or with low resistance, gave offspring with useful resistance. On the contrary, resistant cultivars, such as 'Morgan', 'Dr Molon', 'Sirriner' and 'US 309', gave progeny with very low resistance. Furthermore, the fruit quality of the latter was quite poor. It is concluded that selection for resistance within the more commercially adapted breeding population may be more effective (Bagnara *et al.*, 1996). From this programme, five selections are under advanced tests, including the named cultivar 'Tosca'.

Apple scion breeding programmes

Apple scion breeding programmes for fire blight resistance have not been developed to the same extent as the pear breeding programmes, probably because apples in general are less susceptible than pears. Fire blight resistance has been included in programmes in which scab and mildew resistance are more important. The research stations of Cornell University (Geneva, New York), INRA (Angers, France) and the Institute for Fruit Breeding (Dresden-Pillnitz, Germany), have developed such programmes, with varying degrees of emphasis on fire blight resistance.

At Geneva, New York, each seedling was inoculated with *E. amylovora* and the material was selected, before fruiting, for resistance to fire blight; in recent years, this screening has been applied later in the breeding process. Gardner *et al.* (1980b) found dominant genes which confer resistance in certain highly resistant *Malus* species, *M. robusta* 5 and a selection of *Malus* \times *sublobata*, called 'Novole', which were studied for rootstock breeding. These clones are not completely resistant and a differential genotype \times strain interaction was found with some clones (Norelli *et al.*, 1984, 1986). Certain scab-resistant cultivars and selections are more promising for scion breeding (Aldwinckle and van der Zwet, 1979; Lespinasse and Paulin, 1984, 1990; Korban *et al.*, 1988). Resistance is quantitatively controlled (Gardner *et al.*, 1980b; Korban *et al.*, 1988; Lespinasse and Paulin, 1990; Fischer and Richter, 1996). Cultivars such as 'Liberty' and 'Priscilla' carry a high level of resistance under polygenic control with additive gene effects. Korban *et al.* (1988) studied the segregation of seedlings derived from 16 controlled crosses after fire blight inoculation; the results indicate that there is no evidence of a close linkage between the *Vf* gene from *M. floribunda* 821 coding for scab resistance and genes for fire blight resistance.

At Dresden-Pillnitz, in Germany, parents including *Malus* \times *robusta*, *Malus* \times *floribunda*, as well as several new Pillnitz Pi- and Re- cultivars[®], are used as sources of fire blight resistance. Seedlings are tested in the field; the growing shoots and blossoms are inoculated with a suspension of three highly virulent isolates (Fischer and Richter, 1996). The plant material is scored from 1 (very susceptible) to 9 (no symptoms). Resistance is evaluated after shoot and flower inoculation, since there is no correlation between results obtained with these two assays (Fischer and Schaefer, 1990).

Rootstock breeding

At Dresden-Pillnitz, within the rootstock selection programme, numerous populations from crosses between species and cultivars were evaluated in the greenhouse for their susceptibility to fire blight. Seedlings with low susceptibility were selected from progenies of 'M.4', 'M.11' and *Malus micromalus*, as well as of *Pyrus canescens* \times *Pyrus serrulata* and *P. betulaefolia* \times *P. ussuriensis* (Fischer, 1996).

The apple rootstock breeding programme at the New York State Agricultural Experiment Station (Cornell University), Geneva, New York, has emphasized excellent orchard performance combined with disease resistance over a range of size control since its inception in 1968 (Cummins and Aldwinckle, 1982). Over 350,000 hybrid seedlings have been screened and have resulted in a group of about 20 élite selections, from which four rootstocks have already been released and others are being tested. The programme is continuing actively with breeding and evaluation.

The Geneva programme has used various sources of resistance, including crab-apples and other *Malus* species (Gardner *et al.*, 1980a), hybridized with existing rootstocks (e.g. M.9 and M.26). All seedlings were subjected to challenge from zoospore inoculum of mixed virulent isolates of *Phytophthora cactosorum*. Surviving seedlings were subsequently subjected to multiple inoculations by shoot tip injection with virulent strains of *E. amylovora*, including strains showing differential virulence. Only seedlings showing a high degree of resistance to *E. amylovora* were selected. They were screened for resistance to woolly apple aphid, *Eriosoma lanigerum*, but this was not a determinant factor. Selections were placed in stool beds and further selected. The preselections coming from the stool-bed trial were tested as rootstocks grafted to non-precocious cultivars. The best performers were then subjected to further testing as rootstocks grafted to several cultivars in various locations in the eastern USA. Subsequently, several selections and named rootstocks have been and continue to be tested in the multi-state orchard trials of the NC-140 project.

The four rootstocks released from the programme to date are all resistant to fire blight, but differ in other characteristics (Robinson *et al.*, 1997). Geneva 65 (1992) produces a smaller tree than M.9 and may be sufficiently productive only with vigorous cultivars, with irrigation. Several nurseries have found it difficult to propagate. Geneva 16 (1997) produces a tree with similar size and productivity to that of M.9. It requires further testing before being recommended for large-scale use. Geneva 11 (1993) produces trees of M.26 size, or smaller, with similar productivity. Geneva 30 (1994) produces trees of M.7 size, but with greatly superior precocity and productivity. However, it is rather spiny in the stool bed. CG41, an élite selection that has not yet been released, also looks very promising in the M.9 size range, with excellent precocity and productivity.

Ornamental breeding

Cotoneaster

A breeding programme was developed in Germany in the 1970s, shortly after the introduction of fire blight; this programme resulted in two field-resistant cultivars in the species *Cotoneaster dammeri*. Breeding efforts have also been made with types of the upright-growing species *Cotoneaster franchetii*, *Cotoneaster salicifolius* and *Cotoneaster wateri* (Persiel and Zeller, 1981, 1990); this programme is now discontinued.

Pyracantha

A breeding programme was developed in The Netherlands by Bouma (1990b); 135 crosses were made and 4700 seedlings were tested. Crosses between parents with a high level of resistance, e.g. 'Dart's Red' × 'Buttercup' and 'Dart's Red' × 'Shawnee', resulted in a high percentage of plants without symptoms following inoculation. In France, the *Pyracantha* breeding programme, funded by a commercial association (GIE SAPHYR®), started in 1982 to create new cultivars with resistance to scab and fire blight. In total, 80 progenies and 14,000 seedlings were obtained; about 7000 scab-resistant hybrids were inoculated with a single strain of *E. amylovora*. After multiple inoculations on the successively selected populations, 330 hybrids were planted in the field for selection of ornamental traits (Cadic, 1983, 1984, 1987; Bertrand *et al.*, 1992; Decourtye and Cadic, 1993). From this programme three new cultivars were released: SAPHYR® orange – 'Cadange' and SAPHYR® rouge – 'Cadrou' originating from the cross 'Shawnee' × 'Mozart' (Cadic *et al.*, 1990), and SAPHYR® jaune – 'Cadaune' originating from the cross 'Shawnee' × 'Golden Glow' (Decourtye and Cadic, 1993). These three cultivars are now widely propagated and have replaced the most susceptible cultivars in landscaping in France. During the past 8 years, approximately 1 million plants of these resistant cultivars have been sold in France and in other countries. This breeding programme is continuing to increase the resistance to fire blight, as well as the cold hardiness needed for the North European market.

Somaclonal variation and mutation breeding

Somaclonal variation and mutation breeding can result in improvement of existing cultivars in a shorter period of time than it usually takes to produce a new cultivar by conventional breeding. These breeding methods make possible the selection of new plant types retaining the most favourable properties of the mother cultivar. Furthermore, *in vitro* techniques allow the early selection of new genotypes, thus significantly reducing the number of plants to be tested in the field.

Viseur (1990) regenerated plants from calluses initiated on roots of the pear cultivar 'Durondeau'; *in vitro* screening was performed by artificial inoculation of a virulent strain of *E. amylovora*. Two out of the four somaclonal variants selected with partial fire blight resistance were identified as tetraploids; this ploidy level and the resulting different growth pattern could explain the lower susceptibility to fire blight. The partial resistance of the other two variants, which were diploid, could, according to Viseur (1990), be due to their branching development.

Donovan *et al.* (1994) reported somaclonal variation for fire blight resistance among regenerants from the apple cultivar 'Greensleeves'. Among 270 somaclones regenerated from leaf discs, four were selected on the basis of *in vitro*

shoot and greenhouse inoculations. Chevreau *et al.* (1998) tested the stability for resistance of these four clones *in vitro*, in the greenhouse and under field conditions. Overall results indicated that only one clone was clearly less susceptible than the control. This clone is a 'spur' variant with the same level of ploidy ($2\times$) and the same zymograms as the control; again, the different growth habit could explain the lower susceptibility to fire blight.

Irradiation of *in vitro* explants and subsequent adventitious regeneration has been tested for four cultivars of pear in order to select mutants with a reduced susceptibility to fire blight (Pinet-Leblay *et al.*, 1992). Such a system could be an alternative to the standard *in vivo* irradiation of buds, which usually generates a high frequency of chimeric plants. An *in vitro* screening for resistance to fire blight using a pathogenicity mutant of *E. amylovora* has been developed on detached leaves to select among a mutagenized population, the regenerants showing a hypersensitive reaction (Pinet-Leblay *et al.*, 1996). This programme, initiated at INRA, Angers, is now discontinued and has been replaced by genetic transformation of the pear.

Prospects and breeding strategies

Plant exploration

Plant exploration, particularly in regions where a pathogen has co-evolved with the host, holds great promise for the discovery of useful germplasm for resistance breeding.

Introduction of wild material as seeds allows a more comprehensive sampling of possible genotypes within a species or in the area explored, with a correspondingly greater chance of recovering desirable traits, than does collection of more perishable tissues for asexual propagation. This is well illustrated by the apple exploration programme in North America, Central Asia and China conducted by the USDA Plant Genetic Resources Unit and Cornell University at Geneva, New York (Hokanson *et al.*, 1997). Extensive exploration for pear germplasm in Central and Eastern Europe has been carried out by the USDA Appalachian Research Station, Kearneysville, West Virginia (van der Zwet and Bell, 1990, 1995).

Progeny size for genetic tests

Optimum progeny size may be determined after a preliminary sample estimate of the variance of the population for that character has been obtained. Without such advance knowledge, a good rule is to produce progenies of at least 100 individuals. Estimation of quantitative genetic parameters, for which the variance between families, as well as the variance within families, is needed, will require that a number of families with a wide range of values for fire blight

resistance be produced. For breeding purposes, in which multiple trait selection is to be carried out, much larger progeny sizes are usually desired, with the exact number to be determined by the percentage of seedlings to be discarded by selection for each trait (Dayton *et al.*, 1983).

Selection of parents

Given the long generation time of some fruit crops or ornamental trees, breeding progress may in some cases be enhanced by selecting parents with less than the highest level of resistance available but which retain more nearly acceptable horticultural characteristics; this is the case for apple and pear.

Fire blight resistance present in Asian pear species, *P. pyrifolia* (Burm) Nak., *P. calleryana* Dene. and *P. ussuriensis* Max. is often associated with poor flavour, grittiness, small fruit size or other undesirable characters. Although there appears to be no strict genetic association within hybrid progenies as a whole, the proportion of seedlings with acceptable levels of resistance and fruit quality is low, due to the dominance of species characters. Because a specific type of inheritance pattern characteristic of any of the three species studied (*P. communis*, *P. ussuriensis*, *P. pyrifolia*) was not detected, it was concluded that the same or similar genes for resistance may be present in each species (Bell *et al.*, 1996).

For quantitatively inherited traits, estimates of heritability and of general and specific combining ability may provide an indication of the reliability of the phenotypic scores as indicators of an individual's breeding value (Brown, 1975; Bell *et al.*, 1977). According to Bell *et al.* (1977) and Bagnara *et al.* (1992), the additive sources of variation in pear progeny means exist in sufficient amount (higher than 50%) to enable a reasonable rate of genetic advance in resistance to fire blight. If additive genetic effects predominate over non-additive genetic effects and the environmental variance is low, heritability will be high and parents may be selected on the basis of their own phenotypes. Tests of combining ability may be applied to identify prepotent parents.

The best approach to breeding is multistage selection, aimed at increasing the frequency of favourable genes to resistance. This requires a large number of crosses, instead of a large number of seedlings per cross.

In order to combine fire blight resistance and fruit quality, selection for resistance within the high-quality susceptible cultivars may be the most effective. Selection should thus be applied for many traits at the same time (Bagnara *et al.*, 1996).

Again, with pear, Quamme *et al.* (1990) found a high general combining ability and a low specific combining ability for fire blight resistance. Genetic variance was, therefore, predominantly additive. Genetic advance for fire blight resistance should be obtained by selecting parents on the basis of phenotypic values. Subsequent studies have not supported the hypothesis of a major gene for susceptibility, proposed by Thompson *et al.* (1975), although progenies in

these studies included both 'Bartlett' and 'Doyenné du Comice', used as parents by Thompson *et al.* (1975).

Succession of crosses

Polygenic resistance – that is, resistance conferred by numerous genes of small and more or less equal effect – may be characterized by the method of quantitative genetics. Heritability and combining-ability estimates may be of use to the breeder, if proper precautions are taken in experimental design and the results are interpreted with consideration of the assumptions of the analysis (Bell, 1978).

When resistance is under polygenic control, test or sib-cross generations must be interposed between each back-cross generation in order to recover recessive genes in homozygous form, to identify heterozygous parents or to reconcentrate polygenes. The most feasible method of working with polygenically controlled resistance appears to require development of a pool of individuals, all with as good resistance as can be found by appropriate testing methods and resembling as much as possible the desired cultivar type. Build-up of a polygene system requires that screening procedures be refined to the point that small differences in resistance, conditioned by small numbers of minor genes, can be identified. Intercrossing would provide the large base necessary for horticultural selection.

The search for molecular markers flanking the quantitative trait loci (QTL) for fire blight resistance would facilitate marker-assisted selection. Up to now, no research programme emphasizes the search for molecular markers linked to fire blight resistance; therefore, breeders must continue the breeding efforts already under way, taking into account that host response is inherited in a quantitative fashion and is due to predominantly additive genetic effects, with dominance or epistasis playing only a minor role (Bell *et al.*, 1996). Simultaneous selection for combined high fruit quality or ornamental attributes and fire blight resistance is clearly possible.

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Transgenic Varieties and Rootstocks Resistant to Fire Blight

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A very limited number of apple and pear cultivars are responsible for a large proportion of annual world production. These cultivars are prized by consumers, supermarkets and growers for their appearance, quality, flavour, storability and orchard characteristics. To retain a fruiting cultivar's desirable characteristics and to introduce disease-resistance genes by conventional breeding methods is virtually impossible, because of apple and pear's heterozygosity, long generation time and self-incompatibility, which make back-cross programmes of several generations prohibitively long term and expensive. The use of biotechnology can now overcome these obstacles by introducing resistance genes directly into our current valuable commercial cultivars and thereby transforming them into resistant forms of the same cultivars.

Genes for fire blight resistance can be transferred to both apple and pear using genetic engineering (James *et al.*, 1993; Mourgues *et al.*, 1996). Although no transgenic apple or pear cultivars have been released to date, several programmes are currently using genetic engineering to enhance fire blight resistance. Genetic engineering for resistance to bacteria in plants has been reviewed recently (Düring, 1996; Mourgues *et al.*, 1998a). This chapter will emphasize genes currently being used for resistance to fire blight and current progress with them.

Gene transfer in apple and pear

Gene transfer systems have been developed for apple and pear, but they are genotype-specific. In apple, the scion cultivars transformed include 'Delicious' (Srikandarajah *et al.*, 1994), 'Elstar' (Puite and Schaart, 1996), 'Gala' (Yao *et*

al., 1995; Puite and Schaart, 1996), 'Golden Delicious' (Puite and Schaart, 1996), 'Greensleeves' (James *et al.*, 1989, 1993), 'Jonagold' (De Bondt *et al.*, 1994, 1996) and 'McIntosh' (Bolar *et al.*, 1999). The apple rootstocks transformed include M.26 (Lambert and Tepfer, 1992; Maheswaran *et al.*, 1992) and M.7 (Norelli *et al.*, 1994). Transformation of the pear cultivars 'Conference', 'Doyenne du Comice' and 'Passe-Crassane' (Mourgues *et al.*, 1996), 'Beurre Bosc' (Bell *et al.*, 1998) and 'Vyzhnitsa' (Merkulov *et al.*, 1998) has also been reported.

General considerations in transgene expression and deployment

DNA constructs introduced into plants for enhanced resistance contain a coding region for the protein of interest and several regulatory sequences, which control how, when and where the protein will be expressed in the plant. DNA promoters identify coding regions and allow for initiation of mRNA transcription by RNA polymerase. The 35S promoter from the cauliflower mosaic virus (CaMV) has been used widely in transgenic plants, because it allows for continuous expression, regardless of developmental stage (constitutive expression), in most plant tissues. Duplication of the upstream (5') DNA sequences of the 35S promoter results in elevated levels of transcription (Kay *et al.*, 1987). This promoter is often referred to as the 'enhanced 35S promoter' or the 'double 35S promoter'. However, the term 'double 35S promoter' is a misnomer, since the entire promoter is not duplicated.

Many plant promoters function in specific tissues, during specific developmental stages, or under specific environmental conditions. Targeting gene expression to fire blight-susceptible tissues or during specific developmental stages could be advantageous in providing resistance where and when needed, limiting the amount of transgenic protein in fruit, reducing the plant's energy cost for transgenic protein synthesis and reducing unnecessary exposure of non-target organisms to the protein. To date, most work on transgenic fire blight resistance has focused on identification of effective genes for resistance rather than on the identification of promoters for optimal expression of target genes, but this could be an area of productive research in the future.

An inducible promoter that has been used to study transgenic fire blight resistance and to study resistance in other crops is the proteinase inhibitor II (Pin) promoter from potato, which is wound-inducible in solanaceous plants. In comparing expression of the cecropin B analogue MB-39 in transgenic potato from the Pin promoter, the enhanced 35S promoter and the phenylalanine ammonia-lyase (PAL) promoter of *Arabidopsis*, following inoculation with *Erwinia carotovora* pv. *carotovora*, Huang and McBeath (1997) and Y. Huang (American Phytopathology Society meeting, Rochester, New York, 1997, personal communication) found greater expression and levels of disease resistance from the Pin promoter than the PAL promoter and comparable levels of

expression between the 35S and the Pin promoter. Since wounding and injury are often associated with fire blight infection, expression following wounding could be advantageous. In apple, Pin is expressed constitutively, but expression from the promoter is increased approximately twofold following wounding (Kisung Ko, New York, 1998, personal communication).

Gene expression can be enhanced during translation of mRNA into protein by translational enhancers or by optimization of the coding region sequence. The 40-base, transcribed, untranslated leader sequence from alfalfa mosaic virus (AMV) RNA4 is a translational enhancer that has been shown to result in a 20-fold elevation of β -glucuronidase (GUS) activity in tobacco when constructs contain a native, or unmodified, 35S promoter and a fourfold elevated expression level when the constructs contain an enhanced 35S promoter (Dalta *et al.*, 1993). When attempting to express genes from distant phyla, such as expression of prokaryotic genes in plants, coding regions often need to be altered for optimal translation. Because the genetic code is redundant, with several different three-base codons encoding each amino acid, organisms from different phyla have evolved preferred codon usage for certain amino acids. Resynthesizing genes to adapt their codon usage to those most commonly used in plants can greatly enhance expression in plants. Codon optimization has been important in allowing for efficient expression of both the *Bacillus thuringiensis* endotoxins for insect resistance (Perlak *et al.*, 1991; Van Aarssen *et al.*, 1995; Iannacone *et al.*, 1997) and the green fluorescent protein of *Aequorea victoria* (Rouwendaal *et al.*, 1997) in plants. In addition, prokaryotic genes will often contain polyadenylation-like sequences or cryptic intron splicing sites, which can be recognized by the plant's transcription system, resulting in unstable mRNA. Likewise, modification of these sequences can be important in allowing for efficient gene expression (Green, 1993; Haseloff *et al.*, 1997; Iannacone *et al.*, 1997). However, it should be noted that not all prokaryotic genes require sequence optimization for expression in plants. For example, bacterial genes encoding neomycin phosphotransferase (*nptII*), octopine synthetase (*ocs*), bialaphos resistance (*bar*) and GUS (*uidA*) have all been efficiently expressed in plants without sequence optimization.

Since bacteria multiply in intercellular spaces before causing disease, it has been suggested that expression of antibacterial proteins in intercellular space could be important for their ability to enhance resistance (Düring, 1996). Signal peptides found at the amino terminus of a protein can direct proteins to a target membrane and are removed by cleavage on the *trans* side of the membrane (Verner and Schatz, 1988). Transport of proteins into intercellular space by the eukaryotic secretory pathway results from targeting proteins into the lumen of the endoplasmic reticulum (ER) and later migration of the protein via the Golgi complex toward the cell surface (Iturriaga *et al.*, 1989; Denecke *et al.*, 1990). Denecke *et al.* (1990) found that signal peptides from both animal and plant phyla function in plants. In their experiments, chimeric genes containing either sPR1, the signal peptide of the pathogenesis-related protein 1b of tobacco, or sCEC, the signal peptide of cecropin B (a peptide secreted into the haemolymph

of the insect *Hyalophora cecropia*) functioned equally well in targeting *nptII* to the ER lumen in tobacco.

The benefit of expressing antibacterial proteins in intercellular space is yet to be clearly documented. A potential disadvantage of this approach is that the target protein may be more susceptible to degradation by proteases in intercellular space. As discussed below, transgenic plants expressing analogues of cecropin B, both containing and not containing signal peptides, have been reported to enhance resistance to bacterial pathogens. To test the effect of both signal peptides and translational enhancers on the effectiveness of attacin in enhancing fire blight resistance, Ko *et al.* (1997) have constructed plant transformation vectors containing the attacin E gene (*attE*) under the control of the enhanced 35S promoter: (i) without translational enhancer or signal peptide; (ii) with AMV translational enhancer; and (iii) with both AMV translational enhancer and sPR1 signal peptide.

Another approach that may be advantageous in developing disease-resistant transgenic plants is to express multiple resistance genes that have different modes of action. Engström, P. *et al.* (1984) reported that *Escherichia coli* cells treated *in vitro* with attacin were susceptible to the activity of hen egg-white lysozyme, while non-treated cells were not affected by this lysozyme. Similarly, Jaynes *et al.* (1993) have reported that hen egg-white lysozyme will increase the sensitivity of both Gram-negative and Gram-positive bacteria to the cecropin B analogues, SB-37 and Shiva-1. In addition to the possible synergistic effects of combining different resistant genes, combining genes with different modes of action may increase the durability of resistance, since bacteria would need to simultaneously overcome two different resistance mechanisms. Ko *et al.* (1997) have constructed plant transformation vectors containing *attE* and the T4 lysozyme gene (*e*), cloned both individually and in combination, to test the synergistic effect of combining these genes on fire blight resistance.

Genes to increase fire blight resistance

Genes being evaluated

To date, the genes most extensively used and studied for their effect on fire blight resistance in apple and pear are those coding for cecropins, attacin and lysozymes. The antimicrobial effect of cecropins and attacins was initially identified in insect pupae in response to bacterial infection (Boman and Hultmark, 1987). When pupae of *H. cecropia* are inoculated with pathogenic or non-pathogenic bacteria, they respond by producing several proteins in the haemolymph, which have been identified as isoforms of cecropin, attacin and lysozyme.

Attacins

Attacins are the largest antibacterial molecules found in immunized *Hyalophora* pupae, with a molecular weight of 20–23 kDa. The name ‘attacin’ is derived

from the saturniid tribe, *Attacini*, to which *H. cecropia* belongs (Hultmark *et al.*, 1983). Attacins are comprised of six different isoforms (A–F), which can be fractionated according to their isoelectric point and are divided into a basic group (A, B, C and D) and an acidic group (E and F) (Hultmark *et al.*, 1983). Attacin F is derived by proteolysis of attacin E (Engström, A. *et al.*, 1984). Based upon the isolation of two cDNAs, it was suggested that there are only two attacin genes in *H. cecropia* and that the various isoforms result from post-translational modification of these gene products (Engström, A. *et al.*, 1984). This is supported by the more recent isolation of the attacin gene locus (genomic) from *H. cecropia*, which was found to contain only two functional genes, one corresponding to the acidic and one to the basic attacin cDNA (Sun *et al.*, 1991).

Hultmark *et al.* (1983) found attacin B and E to have *in vitro* bactericidal effects against *E. coli* (lethal concentration 0.3–2 μM), *Pseudomonas maltophilia* (4–15 μM) and *Acinetobacter calcoaceticus* (0.1–1 μM), but activity was not detected (up to 20 μM) against *Enterobacter cloacae*, *Bacillus megaterium*, *Bacillus subtilis*, *Sarcinia lutea*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, and *Streptococcus faecalis*. In contrast, Engström, P. *et al.* (1984) found the effects of attacin to be primarily bacteriostatic against *E. coli*, causing a decrease of growth rate within 1–2 h and complete inhibition of growth at 3–4 h. Cell death occurred only after prolonged exposure (10 h) to high concentrations of attacin (> 8 μM). The higher bactericidal concentrations are well within the range of attacin concentration reported in the haemolymph of induced insects (50–60 μM).

The mode of action of attacin is not well understood. Attacin has no known enzymatic activity. Engström, P. *et al.* (1984) found that treatment of *E. coli* with attacin results in increased sensitivity of the bacteria to β -lactam antibiotics, chicken egg-white lysozyme, and the detergent Triton X-100. Since these compounds are active against the inner membrane or the peptidoglycan of the periplasm and are not normally active against *E. coli* or other Gram-negative bacteria, the authors suggested that attacin caused an increase in the permeability of the outer membrane. Attacin was also observed to cause irregular-shaped cells, irregular patterns of cell division and lysis, which the authors attributed to effects of outer-membrane permeability. Carlsson *et al.* (1991) found that attacin inhibited synthesis of the outer-membrane proteins OmpA, OmpC, OmpF and LamB by interfering with gene transcription. Attacin has also been reported to bind to lipopolysaccharide and to induce the synthesis of certain stress proteins (Axen *et al.*, 1997). However, the induction of stress proteins in response to attacin treatment could be the consequence of its inhibitory effect on cell growth. Contrary to these studies suggesting the outer membrane as the site of action for attacin, attempts to express attacin in *E. coli* expression vectors have shown that low levels of attacin in cytoplasm drastically affect cell growth (Santibanez *et al.*, 1997).

Transformation with an attacin gene is being investigated in apple and pear as a way to enhance fire blight resistance. An *attE* cDNA cloned from *H. cecropia* (Kockum *et al.*, 1984) was cloned into plasmid binary vector pBI121 by Destefano Beltran (1991) for transfer to plants. The vectors included constructs

with *attE* under the control of the 35S promoter or the wound-inducible Pin promoter. Neither construct contains signal peptides to target the protein to inter-cellular space. Aldwinckle, Norelli and co-workers are using these plasmids to study the effect of attacin on fire blight resistance in apple. The plasmid binary vector with *attE* under the control of the Pin promoter was first transferred to an M.7 apple rootstock (Norelli *et al.*, 1994). The integration of *attE* into the apple genome was confirmed by Southern analysis. Northern analysis indicated that the gene was expressed in the transgenic line T1, which was significantly more resistant than M.7 to fire blight in greenhouse tests. Two years of field trials also indicated that attacin transgenic T1 was significantly more resistant than either the parent M.7 or M.7 transformed with the pBI121 vector plasmid (T791) (Momol *et al.*, 1994, 1996). In 1995 field tests, tips of vigorously growing shoots of 3-year-old plants were inoculated by hypodermic syringe with 5×10^9 colony-forming units (cfu) ml^{-1} *E. amylovora* strain Ea273. Resulting areas under the disease progress curve values were T791 : 1201, M.7 : 637 and T1 : 395; and the maximum proportion of the current season's shoot length blighted were 0.55, 0.30 and 0.19, respectively (Momol *et al.*, 1996). Similar results were obtained in 1994 field trials, in which T1, M.7 and T791 were inoculated with strain Ea273 and the differentially virulent strain E4001A (Momol *et al.*, 1994). The attacin gene under the control of the enhanced 35S promoter and the Pin promoter were also transferred to 'Royal Gala' apple (Aldwinckle *et al.*, 1996). In a greenhouse test with artificial inoculation, the 'Royal Gala' transgenic lines that produced the greatest quantities of attacin E, as determined by Western analysis, were those that were most resistant to fire blight (Norelli *et al.*, 1998). More recently, constructs with *attE* under the control of the enhanced 35S promoter alone, or also containing the AMV translational enhancer, or with both the AMV translational enhancer and the sPR1 signal peptide were transferred to both 'Galaxy' apple (Ko *et al.*, 1997) and M.26 apple rootstock (Borejsza Wysocka *et al.*, 1997). Expression of attacin E in both the 'Galaxy' and M.26 transgenic lines has been confirmed by Western analysis (K. Ko and E.E. Borejsza Wysocka, New York, 1998, personal communication) and evaluation of some of the 'Galaxy' transgenics containing attacin E have shown increased resistance to fire blight in growth chamber tests (Aldwinckle *et al.*, 1998).

Eleven transgenic clones of the pear cultivar 'Passe Crassane' were obtained after transformation with EHA101 pFM3002 containing the attacin E gene driven by the 35S promoter. Integration and expression of the transgenes were confirmed by PCR and reverse transcriptase-PCR (RT-PCR), respectively. *In vitro* tests for fire blight resistance were performed by leaf inoculation with a virulent strain of *E. amylovora* CFBP 1430 (5×10^7 cfu ml^{-1}). Infection was rated on a scale from 0 (no symptoms or only inoculated leaf necrotic) to 3 (whole shoot necrotic). The tests distinguished clones with enhanced resistance, 10 days after inoculation of the *in vitro* shoots. Different levels of synthesis of attacin were detected by Western blot analysis. Results from *in vitro* inoculations and Western analysis were in general agreement. Semi-quantitative RT-PCR also indicated higher transcription levels in some resistant clones (Reynold *et al.*, 1999b).

Very limited work has been done studying the effect of attacin on bacterial disease resistance in other crop species. Destefano Beltran transferred the *attE* gene to both tobacco and potato but did not report on the effect of the gene on bacterial disease resistance (Destefano Beltran, 1991). The *attE* gene has been transferred to *Anthurium andraeanum* (Chen and Kuehnle, 1996). When the resulting transgenics were inoculated with the bacterial blight pathogen, *Xanthomonas campestris* pv. *dieffenbachiae*, the *attE* transgenics were reported to show delayed symptom development and reduced pathogen multiplication in comparison with non-transformed controls (Kuehnle *et al.*, 1995).

Cecropins

Cecropins are small peptides, with 35–37 amino acid residues and strongly basic, and comprise three major forms: A, B and D. Comparison of the amino acid sequences of the different forms has revealed a high degree of homology (*c.* 60–80%). The peptides all have a basic amphipathic N-terminal region and a hydrophobic region in the C-terminal part of the molecule. Molecular analysis indicates that the cecropins can form nearly perfect amphipathic α -helices, i.e. cylindrical molecules with charged groups on one longitudinal side and hydrophobic groups on the opposite side (Boman and Hultmark, 1987). Proteins with amphipathic helices are often associated with membranes, and this secondary structure may be of importance for the membrane-disrupting activity of the cecropins (Boman and Hultmark, 1987).

Jaynes *et al.* (1993) synthesized two 38 amino acid peptides, SB-37 and Shiva-1, which were substitution analogues of cecropin B. SB-37 is 95% homologous with cecropin B, with a substitution of valine for methionine no. 11 and the addition of methionine and proline to the NH_2 terminal. Shiva-1 retains only 46% homology with the natural molecule. However, the hydrophobic properties and charge density of the native cecropin B were 100% conserved in the two synthetic peptides. Both of these peptides possess a broad spectrum of antibacterial activity against both Gram-negative and Gram-positive forms. This suggests that the activity of cecropins is not due to a specific active site but rather to the structure and charge of the molecule, consistent with the hypothesis that they disrupt membrane function by direct integration into the membrane.

Jaynes *et al.* (1993) reported the median lethal dose (LD_{50}) of SB-37 and Shiva-1 toward *Clavibacter michiganense* subsp. *michiganense* cells to be 3 and 1.0 μM , respectively, *E. carotovora* subsp. *carotovora* 2 and 0.5 μM , *Ralstonia* (*Pseudomonas*) *solanacearum* 64 and 40 μM , *Pseudomonas syringae* pv. *tabaci* 5 and 2 μM and *X. campestris* pv. *campestris* 0.6 and 0.4 μM . Against *E. amylovora*, both Mourgues *et al.* (1998b) and Norelli *et al.* (1998) found that native cecropin B had higher *in vitro* activity than its analogues SB-37 or Shiva-1. By exposing cells of *E. amylovora* strain Ea273 in phosphate buffer to various concentrations of the peptides for 1 h, Norelli *et al.* (1998) found LD_{50} concentration of 0.2 and 3 μM for cecropin B and Shiva-1, respectively. When *E. amylovora* strain CFBP1430 was grown in liquid King's medium B, containing dilutions of

various peptides, the minimal inhibitory concentrations that prevented growth were 5 μM , 15 μM and > 50 μM for cecropin B, SB-37 and Shiva-1, respectively (Mourgues *et al.*, 1998b).

Plant cells are also sensitive to cecropin but generally at higher concentrations than are bacterial cells. Nordeen *et al.* (1992) determined the lethal concentration of cecropin SB-37 for protoplasts of 11 cultivars of tobacco, tomato, potato, sugar beet, soybean and sweet potato and for 14 strains of nine different bacterial pathogens of these plants. Lethal concentrations ranged from 4.5 μM for tomato to 41 μM for sugar beet, and for the bacterial cells from 0.1 μM for *P. syringae* pv. *glycinea* to 4.5 μM for *P. syringae* pv. *tomato*. Based upon the greater sensitivity of bacterial pathogens, the authors concluded that it may be feasible to protect certain plants against these pathogens by the introduction of a modified cecropin gene.

Due to the small size of cecropin, synthetic genes have frequently been used in developing cecropin transgenic plants. Synthetic coding regions have been constructed from synthesized, overlapping DNA oligonucleotides, which are annealed *in vitro*, ligated and cloned into an appropriate vector (Destefano Beltran, 1991; Hightower *et al.*, 1994). PCR has frequently been used in subsequent cloning of altered constructs using the original synthesized coding regions as template (Huang and McBeath, 1997; Ko *et al.*, 1997).

Variable results have been obtained expressing cecropins in transgenic plants for enhanced resistance to bacterial diseases (Düring, 1996). This variability is probably due to differences in the transgene constructs used, their effectiveness in specific host-pathogen systems and the methods used to determine host resistance. Cecropin constructs used in transgenic plants have varied in the use of native versus novel coding regions, the inclusion of signal peptides and the types of promoters used. In general, novel cecropin analogues have been more effective than native cecropin A or B. The best results have been obtained with the Pin promoter of potato, but it is difficult to draw any conclusions regarding either the most effective promoters to use or the benefit of using signal peptides. To date, most of the work with transgenic plants has been done with tobacco and potato.

Jaynes *et al.* (1993) found that expression of the cecropin Shiva-1 driven by Pin in transgenic tobacco conferred enhanced resistance to bacterial wilt caused by *R. solanacearum*, with R1 seedlings showing delayed wilt symptoms and reduced disease severity. R1 seedlings of plants transformed with the SB-37 cecropin analogue under the control of the enhanced 35S promoter were not significantly more resistant than non-transgenic control plants. Neither SB-37 nor Shiva-1 constructs contained signal peptides, and the localization of the peptides was not studied.

In contrast, attempts to express native cecropin A or B in transgenic plants has not resulted in significant decreases in bacterial disease (Hightower *et al.*, 1994; Allefs *et al.*, 1995). Allefs *et al.* (1995) attempted to express cecropin B under the control of the enhanced 35S promoter in potato using a gene construct which contained the α -hordeothionin signal peptide. Northern analysis

indicated that the cecropin B gene was transcribed in transgenic plants, with the highest transcript levels being approximately 0.6% of total mRNA. However, no cecropin B could be detected in transgenic plants by Western analysis and the plants showed no increase in resistance to *E. carotovora* subsp. *atroseptica*. Similarly, Hightower *et al.* (1994) expressed cecropin A under the control of the 35S promoter with the cecropin B signal peptide in tobacco. Northern analysis indicated that the cecropin A gene was expressed in transgenic plants and cecropin was detected using an ELISA technique, but no increase in resistance to *P. syringae* pv. *tabaci* could be detected.

A possible explanation for the failure of native cecropins to be detected in transgenic plants or to significantly enhance bacterial disease resistance is that these peptides are rapidly degraded in plant tissues, particularly in intercellular space. Several researchers have reported that cecropin B is rapidly degraded, with a half-life in intercellular fluids ranging from 3 min in potato to about 25 h in rice (Mills *et al.*, 1994; Owens and Heutte, 1997). Similar results were obtained in pear. Antibacterial activity of cecropins was abolished when they were pre-incubated in crude pear intercellular washing fluids, and degradation of cecropins after 2.5 h incubation in crude pear intercellular washing fluids was also demonstrated (Mourgues *et al.*, 1998b). Proteases in intercellular fluids have been implicated as the cause, since degradation can be lessened or stopped by boiling intercellular fluids or by the addition of protease inhibitors (Mills *et al.*, 1994; Allefs *et al.*, 1995). Owens and Heutte (1997) found that the substitution of one base pair in cecropin B was associated with diminished degradation by leaf intercellular fluids. The cecropin B analogue MB-39 had a longer half-life in the intercellular fluids of nine out of ten species and, overall, the half-life averaged 2.9 times greater than that of cecropin B. Although MB-39 contained minor changes to both the N and C termini of the peptide, analysis of peptides produced by endopeptidase activity indicated that a substitution of valine for methionine at residue 11 of cecropin B was primarily responsible for the increased stability. In contrast to the work of Hightower *et al.* (1994), using native cecropin B, Huang and McBeath (1997) demonstrated that transgenic tobacco containing the cecropin analogue MB-39 had increased resistance to *P. syringae* pv. *tabaci*. MB-39 was expressed in tobacco from the Pin promoter and contained an α -amylase signal peptide. Although this work possibly explains why native cecropin B constructs failed to show activity, it does not explain why Jaynes *et al.* (1993) did not observe activity using the SB-37 construct, since both MB-39 and SB-37 contain the same valine for methionine-11 substitution (Huang and McBeath, 1997).

The cecropin B analogues SB-37 and Shiva-1 have been transferred to 'Royal Gala' apple and M.7 apple rootstock (Aldwinckle *et al.*, 1996; Norelli *et al.*, 1996). The Shiva-1 construct is controlled by the enhanced 35S promoter and contains no signal peptide. Various SB-37 constructs have been used with either the Pin promoter or the enhanced 35S promoter. The gene has been expressed without signal peptide or with either the sPR1 signal peptide from tobacco or the sCEC signal peptide from *H. cecropia*. In 1997, approximately 120

field-grown plants of 13 'Royal Gala' transgenic lines containing the cecropin SB-37 transgene were evaluated for their resistance following inoculation of vigorously growing shoot tips with *E. amylovora*. In this test, several of the transgenic lines developed less disease than 'Royal Gala', but only transgenic line T245 (12% shoot length infected) was significantly more resistant than 'Royal Gala' (67%). Leaf tissue was harvested from plants 48 h after inoculation and the level of cecropin expression was determined by ELISA. In general, correlation between the amount of cecropin SB-37 detected in tissue and the level of resistance was not significant, but T245, which was significantly more resistant than 'Royal Gala', had the highest level of SB-37 expression (630 pg μg^{-1} soluble protein). In addition, the cecropin B analogue MB39, under the control of the wound-induced osmotin promoter from tobacco, has been transferred to 'Royal Gala' with the goal of increasing fire blight resistance (Liu *et al.*, 1998).

The pear cultivar 'Passe Crassane' was transformed using *Agrobacterium* strain EHA101 with the plasmids pFM3003, pFM3004 and pFM3005, which contained the SB-37 gene under the control of the 35S promoter. In pFM3003 and pFM3005, the SB-37 gene was fused to the cecropin B and PR1 signal peptides, respectively. An additional plasmid, pFM3008, containing the Shiva-1 gene fused to PR1 signal peptide, under the control of 35S, was also used. DNA from 26 putative transgenic clones exhibited PCR amplification of a fragment of the SB-37 or Shiva-1 gene. Transcription was observed in all clones by RT-PCR. We were not able to detect SB-37 or Shiva-1 antigen by Western blotting with horseradish peroxidase-labelled antibodies. Fire blight susceptibility was quantified 10 days after leaf inoculation of *in vitro* shoots. Inoculations led to the onset of severe necrosis in cultivar 'Passe Crassane' after 3 days. Transgenic clones with reduced symptoms could be distinguished after 10 days (Reynold *et al.*, 1999a, c).

Lysozymes

Lysozymes are bacteriolytic enzymes, which have been characterized from phage, bacteria, fungi, plants and animals (Jollès and Jollès, 1984). They cleave the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the bacterial peptidoglycan, resulting in weakening of the cell wall and leading to eventual lysis of the bacteria (Phillips, 1996; Düring, 1996). Some lysozymes also have chitinase activity, resulting from random hydrolysis of 1,4- β -*N*-acetylglucosamine linkages in chitin (Osserman *et al.*, 1974). Both hen egg-white lysozyme and bacteriophage T4 lysozyme are being investigated in transgenic plants for enhancement of disease resistance. Although these two lysozymes have a completely different primary structure, there is a high degree of similarity in their secondary structure (Matthews *et al.*, 1981). In direct comparison of the *in vitro* activity of T4 and hen egg-white lysozymes, Düring (1994) found that T4 lysozyme was active against both *R. solanacearum* and *E. carotovora* subsp. *atroseptica*, while hen egg-white lysozyme was active only against *E. carotovora* subsp. *atroseptica*.

Düring *et al.* (1993) transformed the Z2 genotype of potato with a chimeric

T4 lysozyme gene, consisting of the α -amylase signal peptide of barley and the bacteriophage T4 lysozyme gene, under the control of the 35S promoter. The same chimeric lysozyme gene was previously shown to secrete T4 lysozyme protein to the intercellular space of transgenic tobacco (Hippe *et al.*, 1989). T4 lysozyme transgenic potato tubers were more resistant to maceration by *E. carotovora* pv. *carotovora* than non-transgenic controls, and transgenic plants were also more resistant in a greenhouse sprouting test after inoculation with *E. carotovora* pv. *carotovora* (Düring *et al.*, 1993). More recently, the 'Désirée' cultivar of potato has been transformed with an optimized plasmid binary vector (pSR8-36) containing the same chimeric T4 lysozyme. The resultant 'Désirée' transgenics have likewise shown increased resistance to maceration and improved sprouting following inoculation with *E. carotovora* pv. *carotovora* (Düring, 1996).

Hanke *et al.* (1998) have transferred the T4 lysozyme gene, using pSR8-36, to 'Pinova' apple, a new apple cultivar developed at Pillnitz, Germany. Transfer of the gene was confirmed by ELISA for NPTII protein and by PCR to confirm the presence of the lysozyme gene. Currently, the plants are being propagated for evaluation of fire blight resistance and are being studied for expression of T4 lysozyme (Viola Hanke, Dresden-Pillnitz, Germany, 1998, personal communication). Ko *et al.* (1997) cloned the pSR8-36 chimeric T4 lysozyme gene containing the α -amylase signal peptide into pBIN19, under the control of the enhanced 35S promoter, with the AMV translational enhancer. Ko *et al.* (1997) also combined this chimeric T4 lysozyme gene (enhanced 35S, AMV translational enhancer, α -amylase signal peptide, T4 lysozyme, *nos* terminator) with *attE* under the control of Pin promoter. This new T4 lysozyme construct and the combined T4 lysozyme/attacin E gene construct have been transferred to 'Galaxy' apple (Aldwinckle *et al.*, 1998; Ko *et al.*, 1999) and the M.26 rootstock (Borejsza Wysocka *et al.*, 1997). Similarly to the work by Hanke *et al.* (1998), transformation was confirmed by ELISA for NPTII protein and by PCR, and the transgenics are being evaluated for fire blight resistance and expression of T4 lysozyme. Some T4 lysozyme transgenics of 'Galaxy' have shown increases in fire blight resistance in preliminary growth chamber tests (Kisung Ko, New York, 1998, personal communication).

Other potential genes

Several other antimicrobial proteins are known to occur in plants and animals, and could potentially be used in transgenic plants for enhanced fire blight resistance (Holz and Stahl, 1995). These include tachyplesin (Allefs *et al.*, 1996), thionin (Carmona *et al.*, 1993; Florack *et al.*, 1994), magainin (Zasloff, 1987) and other synthetic peptides (Powell *et al.*, 1995).

Alternatively, other plant defence responses to pathogen infection could be enhanced by genetic engineering. For example, the production of active oxygen species, including hydrogen peroxide, is part of the plant defence response, and elevation of active oxygen species in transgenic plants is a possible means of

enhancing disease resistance (Wu *et al.*, 1995). Glucose oxidase, an enzyme found in a number of bacteria and fungi, catalyses the oxidation of β -D-glucose by molecular oxygen, yielding gluconic acid and hydrogen peroxide (Frederick *et al.*, 1990). Wu *et al.* (1995) expressed the glucose oxidase gene of *Aspergillus niger* in transgenic potato and observed elevated levels of hydrogen peroxide in both leaves and tuber tissues. Transgenic potatoes with elevated hydrogen peroxide levels also exhibited strong resistance to soft rot disease caused by *E. carotovora* subsp. *carotovora* and enhanced resistance to potato late blight caused by *Phytophthora infestans*. Other possible means of enhancing plant defence responses include expression of pathogenesis related genes or genes encoding enzymes to synthesize unique or new phytoalexins (Hain *et al.*, 1990, 1993).

Based upon recent advances in our understanding of the molecular biology of fire blight biology, pathogen-derived resistance is a potential strategy for engineering resistance to fire blight. Harpins, proteinaceous elicitors of the hypersensitive response, have been identified and characterized and the genes encoding them have been cloned and sequenced from *E. amylovora* (Wei *et al.*, 1992; Kim and Beer, Chapter 8). Solutions of pure harpin elicit the hypersensitive response in non-hosts in a manner that is indistinguishable from that elicited by metabolizing bacteria, and mutants of *E. amylovora* incapable of producing harpin are not pathogenic on pear or apple. The possibility of using the *hrpN* gene encoding harpin to engineer fire blight-resistant apple is currently being evaluated in the laboratories of Herb Aldwinckle and Jay Norelli, and of Steven Beer, using two different approaches. One approach is to express a low level of harpin in transgenic plants to induce systemic acquired resistance. Treatment of tomato seed with harpin has been shown to induce resistance to *R. solanacearum* (Qui *et al.*, 1997). In orchard tests, harpin applied to apple at the pink and full-bloom stages resulted in a significant reduction in the development of blossom blight (Momol *et al.*, 1998), indicating that apple can be induced to resist *E. amylovora* by treatment with the proteinaceous product of *hrpN*. The other approach is to engineer a system where, upon infection, plants would produce harpin in sufficient quantities to elicit the hypersensitive response and induce resistance to *E. amylovora*. For this approach to work, *hrpN* would need to be placed under the control of a promoter that is induced specifically by *E. amylovora* infection. Currently, the pathogenesis-related promoter of the *prp1-1* gene of potato is being evaluated for this purpose.

Another possible approach is to alter host metabolism to favour resistance. Suleman and Steiner (1994) have proposed a model to explain the fire blight susceptibility of tissue based upon sorbitol concentration. According to the model, increases in sorbitol concentration result in increasingly negative solute potentials, which have negative effects on the growth of *E. amylovora*, thus rendering these tissues more resistant. A key enzyme in the synthesis of sorbitol, sorbitol-6-phosphate dehydrogenase (S6PDH), has been cloned from apple (Kanayama *et al.*, 1992; Kanayama and Yamake, 1993) and its expression in transgenic tobacco was correlated with sorbitol synthesis and concentration (Tao *et al.*, 1995). Abhaya Dandekar (California, 1998, personal communica-

tion) has produced transgenic apple trees with suppressed and elevated levels of sorbitol synthesis by expressing the S6PDH gene in either the antisense or the sense orientation. Evaluation of these trees for their fire blight susceptibility will be a good test of the Suleman and Steiner (1994) model and may lead to the identification of resistance.

The technology for efficient transformation of apple and pear has now been developed, and transgenic plants and fruiting trees of apple, at least, can be obtained reliably and relatively quickly. Some transgenic lines of apple have shown a high level of resistance to fire blight in field trials. Orchard trials for fruit and tree characters are under way. Thus the technical hurdles to the use of genetic engineering to obtain fire blight-resistant cultivars are rapidly being crossed. However, the commercialization of such transgenics for the practical benefit of pear and apple growers will be complicated by regulatory requirements and licensing.

In the USA, the federal agencies Food and Drug Administration (FDA), Environmental Protection Agency (EPA) and Animal and Plant Health Inspection Service (APHIS) of the US Department of Agriculture, are mandated to ensure that all concerns regarding food safety, environmental hazards and risks to agricultural crops (e.g. producing noxious weeds) are fully addressed. Transgenics containing compounds that are active against pathogens and insects, with some exemptions, are regulated as pesticides, and have to fulfil the requirements of pesticides for registration.

Transformation technology and the genetic material (genes, promoters, translational enhancers, selectable markers, etc.) used to create transgenic plants are covered by numerous patents, for which licences must be obtained for commercialization. Although it is not trivial to negotiate licences with all the patent-holders involved, we are optimistic that mutually satisfactory arrangements can eventually be achieved.

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Fire Blight Risk Assessment Systems and Models

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Introduction

Theories and hypotheses are modified more often than they are discredited. A realistic methodology must be one that allows for repair as readily as for refutation.

(Medawar, 1969)

Fire blight risk assessment systems are working hypotheses based on a combination of knowledge, speculation and trial and error (Steiner, 1996). For people with experience of fire blight, these systems give valuable guidance on which to base good judgements of action, in spite of problems associated with their development and evaluation.

Fire blight is a complex disease. There is no curative bactericidal agent available for control and no protective agent is systemic (Psallidas and Tsiantos, Chapter 11). The best protective agent, streptomycin, can no longer be used in many areas, because the pathogen has developed resistance to it (Jones and Schnabel, Chapter 12). It affects a variety of pome fruit hosts, including pears, apples, hawthorns, pyracanthas and cotoneasters. The spread of infections from one species to another species can be important. Furthermore, fire blight occurs in a variety of climatic areas, ranging from semi-arid to areas with warm wet springs and spring and summer storms (Bonn and van der Zwet, Chapter 3). Fire blight risk assessment is made even more difficult by the fact that flowers, as well as young leaves and fruits, are susceptible to infection and often killed. Subsequent stem invasion can destroy branches or even whole trees. Inoculum may be spread by rain, wind or insects or by pruning and budding tools (Thomson, Chapter 2). Storm damage can greatly increase chances of infection. This explains why the list of the principal risks requiring assessment, summarized

in Box 15.1, is so long. For comparison, the principal risks for the fungal disease apple scab (*Venturia inaequalis*) are outlined in Box 15.2.

Fire blight is a sporadic disease in space and time, which makes epidemiological studies and spray trials difficult to plan and the relative value of different approaches to risk assessment difficult to judge. In years of high disease incidence, there can be great differences in incidence between hosts, between cultivars and between locations. These differences largely depend on whether or not a host or a cultivar was in flower when weather-related risks were high. Such problems mean that statistical approaches to system and model design and their evaluation are rarely possible. Thus, publication in major English-language journals is rare.

Nevertheless, useful guidance has now emerged for the optimal timing of protective spray applications during bloom and for the optimal timing of searches for signs of new disease to ensure early surgical control. This guidance

Box 15.1. Principal factors in fire blight risk assessment.

Blossom blight (primary, late and secondary blossom) risks

Inoculum sources: overwintering cankers (twig and stem); colonized open flowers; new disease; cryptic blight; other hosts

Inoculum potential (difficult to assess): past disease; late season blossom and shoot infections and late stem invasion (unsealed cankers); cankers active from bud burst onwards; times of inoculum release; doses of the pathogen in colonized flowers; inoculum from new disease

Localized inoculum spread: by rain

Random inoculum spread: by insects, especially between open flowers and between trees and plantations; dry winds (if strands are present)

Infection: flower susceptibility; inoculum potential; flower wetting (dew, mist or rain); immediate entry via nectarthodes; entry via wounds (frost, storm or insect damage)

High incidence risks: many overwintering cankers; high temperatures during bloom, especially at full bloom (high flower colonization rates and insect spread rates)

Green tissue blight (flowers at green cluster stage; young leaves, shoots and fruits) risks

Inoculum sources, potential and spread: see blossom blight

Infection: high inoculum dose spread by rain; low doses require wounds (frost, hail, strong winds or insects)

High incidence risks: inoculum from earlier disease on host or other host; damaging storms with wind-blown rain; damaging insects

Disease development rates

Following direct infections: highly temperature-dependent

Further stem invasion: host-, temperature- and soil-moisture-dependent

Box 15.2. Principal factors in apple scab (*Venturia inaequalis*) risk assessment (from Xu *et al.*, 1995; MacHardy, 1996).

Inoculum sources: ascospores on leaf litter; conidia on twig pustules, bud scales and new leaf lesions

Inoculum potential: past disease incidence, late summer infections; disease management; weather (postharvest, late winter and early spring); overwintering leaf litter

Inoculum spread: rain splash

Infection: Leaf, calyx and fruit susceptibility and maturity; inoculum potential; Infection Period (for spore germination and hyphal penetration of leaf surfaces); a surface wetness period whose length depends on temperature (shorter at higher temperatures) (compare the immediate entry into host tissue by the fire blight pathogen at the time of wetting)

Leaf lesion incidence: host susceptibility; inoculum potential; number of infection periods; favourability of the infection period

Note. Fungicidal agents are available for control of disease after infection, as well as protective agents for use before infection.

is often applicable to different hosts and in different climatic areas, although adjustment might be needed.

This chapter aims to show how approaches to fire blight risk assessment evolved and to give guidance on the principles on which they are based and on their applications.

For the benefit of non-English-speaking people and those unfamiliar with specialist terms, terminology has been kept as simple as possible. Furthermore, definitions and alternative terms used in other publications are presented in Table 15.1. The term 'predictive system' has been avoided, because it can give rise to false expectations.

Field risks

Overall disease management is outside the scope of this chapter – it is treated by Steiner (Chapter 17) – but risks beyond those listed in Box 15.1 have to be considered. Before streptomycin was available for blossom protection, reliance was placed on orchard management to limit disease incidence and severity. Some key factors not included in Box 15.1 are listed in Box 15.3. Most of these are still included in current publications used for advisory purposes in different countries (e.g. van der Zwet and Beer, 1995).

Field risks are too often ignored. Failure to assess such risks correctly can lead to over- or underestimation of weather-related risks. For this reason, some

Table 15.1. Terminology.

Used in this chapter	Definitions or alternative terms
Blossom blight	Direct or indirect blossom infections
Shoot blight	Direct or indirect shoot infections; twig blight
Fruit blight	Direct or indirect fruit infections
Stem blight	Bark invasion following earlier infections
Direct infections	Entry at terminal part of organ from external sources
Indirect infections	Entry via the base of the organ from earlier disease; movement in bark tissue to terminal parts
Sealed cankers	Determinate cankers
Unsealed cankers	Indeterminate cankers
Spring canker activity	Systemic canker activity (SCA) ^a
Movement from cankers (indirect infections)	Canker blight symptoms (CBS) ^a
Damage-induced blight (frost, hail, wind)	Trauma blight symptoms (TBS) ^a
Flower colonization	Epiphytic, saprophytic growth
Cryptic disease:	Hidden disease:
external	small atypical lesions
internal	endophytic
Ooze (droplets or strands)	The pathogen plus polysaccharide
Inoculum potential (IP):	Available inoculum:
from all sources	ooze or invisible
in colonized flowers	relative epiphytic inoculum (infection) potential (EIP) ^a
Infection risk (IR) day	Infection event ^a
Disease development period (D-period)	Incubation period (I-period)
Other hosts	Hawthorns (<i>Crataegus</i>), <i>Sorbus</i> spp., pyracanthas, cotoneasters, etc.
Surgical control	Cutting out diseased parts or digging up tree
System	Model

^a Terms used in the Maryblyt model (Steiner, 1990a, b).

risk assessment systems incorporate a broad assessment of some field risks. With experience, the grower and adviser will learn which risks are most important in their location and under their conditions. A suggested list of field records useful for field risk assessment is given in Box 15.4.

A distinction must be made between pears, apples and other hosts and between young and mature trees, because risks can sometimes differ. Records of the start and end of bloom are essential and should cover overlap between hosts (e.g. pear and apple; apple and hawthorn; pyracantha and cotoneaster and pear secondary blossom). Different forms of secondary blossom on pear need to be considered (Deckers, 1996). On apple, late flowers on 1-year wood

Box 15.3. Some factors considered significant in fire blight management before 1940.^a

Host susceptibility

- Wide variation between host species and cultivars
- Susceptibility is associated with rapid growth
- Rapid growth is associated with: young trees; excess nitrogen or irrigation; other soil factors; some pruning practices

Inoculum production and spread

- Unsealed stem cankers are most likely to overwinter
- Overwintering twig cankers high in the tree
- Eradication of all cankers is unlikely
- Highly susceptible trees produce the most ooze
- Trees near beehives are worst affected by blossom blight
- Contaminated pruning tools
- Infected nursery material

Infection risks

- Late spring flowers and secondary blossom are major hazards
- Infection is rapid if leaf traces are exposed during storms
- Entry can occur via leaf axils
- Water shoots and blossoms allow direct entry to main stems
- Collar blight is associated with burr knots and root suckers

Stem invasion

- Invasion is via living bark tissue
- It may proceed rapidly in a narrow line
- It may continue until leaf fall and then further in subsequent years, sometimes without external signs of disease

^a Information for this box came from papers cited by Baker (1971) and van der Zwet and Keil (1979) and from other early publications. Many of the risks listed in Box 15.2 were well understood before 1940.

can be important. Records of storm damage during periods of rapid shoot growth are also essential. Often overlooked are indirect infections in spring (Billing *et al.*, 1974; Billing, 1996), where stem invasion of blossoms or shoots occurs via their base from overwintering cankers (referred to as 'canker blight' by Steiner, 1990b). Their incidence is usually low.

Oozing stem cankers are an obvious source of inoculum in spring, and twig and stem cankers high in trees can be a special danger. In some areas, in spite of diligent searches, ooze is rarely seen in spring. Ooze commonly emerges after midnight (Eden Green, 1972) and can be sucked back as humidity levels fall (E. Billing, unpublished records), so inspections are best made before sunrise. There is a suspicion that other less obvious sources of inoculum are sometimes important.

Box 15.4. Suggested field records.

1. Dates for pear and apple cultivars and other hosts

Bud break	<input type="text"/>	Rapid shoot growth periods	<input type="text"/>
First open flowers	<input type="text"/>	Rapid girth expansion	<input type="text"/>
Full bloom	<input type="text"/>	Late shoot growth	<input type="text"/>
Petal fall (80–90%)	<input type="text"/>	Leaf fall	<input type="text"/>
Late flowers	<input type="text"/>		
Secondary blossom	<input type="text"/>		
Autumn blossom	<input type="text"/>		
First blossom blight	<input type="text"/>	Storm damage	<input type="text"/>
First shoot blight	<input type="text"/>	Insect damage	<input type="text"/>
First fruit blight	<input type="text"/>	Other damage	<input type="text"/>
Spray dates	<input type="text"/>	Irrigation dates	<input type="text"/>

2. Disease incidence (high, medium, low, none)

Pre-bloom blight	<input type="text"/>	Sealed stem cankers	<input type="text"/>
Blossom blight	<input type="text"/>	Unsealed cankers	<input type="text"/>
Shoot blight	<input type="text"/>	Twig cankers	<input type="text"/>
Fruit blight	<input type="text"/>	Spring canker activity	<input type="text"/>
Stem blight	<input type="text"/>	Indirect infections	<input type="text"/>
Nearby orchards	<input type="text"/>	Nearby other hosts	<input type="text"/>

The full list is formidable but valuable for good risk assessments and also when evaluating and comparing different systems and models and protective and control measures.

However, various reports suggest that a single oozing canker can supply enough inoculum for epidemic blossom blight to develop if conditions favour rapid flower colonization and rapid inoculum spread between flowers by insects (Thomson, Chapter 2).

The evolution of risk assessment systems and models

Although Bordeaux mixture was used for fire blight control from the 1920s onwards, before 1955 there was no weather-based advice for the timing of spray applications, for judging infection risk (IR) days or for timing of searches for signs of new disease. As well as risks listed in Box 15.3, some general

assumptions were that disease was favoured by moist soil and warm humid weather; the same conditions and cloudy days favoured ooze production, especially in spring, 'when the trees are gorged with sap' (Waite, 1896); hot or cool, dry, sunny weather and dry winds discouraged activity. Rain was considered an important agent for spreading inoculum but, during bloom, spread by insects could be rapid. In addition, flowers were said to be colonized rapidly by the pathogen in warm, humid weather (Thomas and Ark, 1934). An association between fire blight and thunderstorms was noted early: 'Farmers thought that trees were struck by lightning' (Denning, 1794). The importance of storm damage and heavy rain were understood later.

With the introduction of streptomycin as a protective agent, routine applications throughout bloom proved costly and often wasteful and likely to encourage the development of streptomycin resistance (Jones and Schnabel, Chapter 12). Risk assessment systems were developed to ensure optimal timing of applications in relation to risks of infection. In areas where protective agents were not used during bloom, optimal timing of searches for signs of new disease was the main aim.

The development of systems and models in the USA

Degree-days above 18.3°C (Mills, 1955; Luepschen *et al.*, 1961; Powell, 1965)

Mills (1955) was aware that warmth plus wetness favoured blossom blight, so he studied the relationship between these factors and the severity of apple blossom blight in the Lake Ontario region of New York, where rainfall is frequent. The best correlations with disease severity came when he summed the degree-days (DD) (DD = equals the number of degrees over the base temperature during 1 day) where there was a trace or more of rain, using 21.1°C or 18.3°C as the base. When 26.7°C was used as the base, rainfall appeared to be unnecessary. Mills suggested that, 'on a limited trial basis', the first streptomycin sprays should be applied on the first day when temperatures above 18.3°C with rain or high humidity were forecast. It was not suggested that this advice would be applicable outside the area examined.

Parker *et al.* (1956) noted that, in summer, optimum rainfall for tree growth favoured progression of existing infections. Luepschen *et al.* (1961) observed in greenhouse experiments that pear blossom infection occurred with daily mean temperatures of 13.9°C. In the orchard, such mean temperatures would usually mean a daily maximum of 18.3°C or more. An IR day was defined as one where the maximum temperature was 18.3°C or more with rain on that day or with a mean percentage relative humidity (%RH) of 70 or more if the warm day followed rain. They noted that severe blight could occur with late flowers opening during petal fall. For good control, using streptomycin, applications needed to be made during or before the first IR day, with repeat sprays later, if necessary.

The emphasis in Illinois (Powell, 1965) was on the importance of adequate inoculum potential (IP) and on host susceptibility. Ooze-producing cankers were most likely on susceptible hosts growing vigorously and early spring infections were commonly seen near cankers. Conditions favouring blossom blight were: 17 DD above 18.3°C between the last pre-bloom frost and early bloom for adequate inoculum levels; maximum temperatures in early bloom of 21.1–26.7°C and less than 30°C (the optimum temperature range for growth of the pathogen); adequate pre-bloom rainfall and light (not excessive) rains and high humidity (mean %RH 70 or more) in early bloom.

The mean temperature line (Thomson *et al.*, 1982)

Risk assessment methods used in the wetter eastern states of the USA and in England did not offer useful guidance for the timing of protective spray applications in California, where rain is often infrequent during pear blossoming. A new approach was based on knowledge gained from monitoring the presence of the pathogen on pear blossoms (Miller and Schroth, 1972; Thomson *et al.*, 1975). Later, Thomson (1986) showed that epiphytic populations occurred mainly on stigma surfaces of pear flowers and of apple, pyracantha, cotoneaster and hawthorn flowers. Monitoring flower populations is laborious, so it was important to discover the relation of temperature to the occurrence of the pathogen in flowers. In a 4-year study (Thomson *et al.*, 1982), the pathogen could not be found in flowers before the mean temperature exceeded a line drawn from 16.7°C on 1 March to 14.4°C on 1 May, so this line was used as a simple guide to the timing of the first protective spray application, with the aim of preventing the build-up of pathogen populations in flowers. If the mean temperature line was crossed before or during a period of heavy bloom, severe blight was likely, especially if rain occurred during a warm period.

However, in some north coast counties of California, where there is sometimes a large difference between day and night temperatures, the pathogen was not detected until long after the mean temperature line was crossed. In Michigan and New York, a simple relationship between mean temperatures and epiphytic populations in apple blossom was not found (Sutton and Jones, 1975; Beer and Opgenorth, 1976). It appeared, therefore, that the approach might not be widely applicable.

The mean temperature line (Thomson *et al.*, 1982) is still used for timing spray applications in many orchards in California, Washington and Oregon and in Utah, where a single mean temperature of 15.6°C is now preferred (S.V. Thomson, Utah, personal communication). The aim is to prevent early flower colonization.

The degree-hour model (Zoller in van der Zwet *et al.*, 1988; Gubler *et al.*, 2000)

Although a mean temperature of 15.6°C was of value for predicting the initial presence of the pathogen in pear flowers (Zoller, 1978), it provided too little

information on subsequent risks during bloom. A study was therefore made of the relationship between degree-hour (DH) sums above 18.3°C (1 DH is counted when the temperature has been 1°C above the threshold for 1h) and the presence of *Erwinia amylovora* in blossoms (Zoller, 1978; Zoller and Sisevich, 1979). When 200 DH had accumulated, it was found that 10% of blossom samples were colonized by the pathogen. The percentage increased faster as the DH sum increased but, if temperatures fell below 18.9°C for 3 consecutive days, the percentage fell to zero.

The relationship between blossom blight incidence and weather was then studied from early bloom to 30 days past full bloom. DH above 18.3°C were accumulated prior to periods with rain or to periods with high humidity (%RH 90 or more) when the temperature was at least 13.9°C. There was a strong correlation between the DH sum (early bloom to 15 days past full bloom) and the later incidence of new blight if inoculum potential from overwintering cankers was considered. The model is used to guide timing of applications of protective agents at increased frequency with increasing DH sums, especially during periods of heavy bloom. Different threshold DH sums are recommended for different climatic areas in California. The importance of removing overwintering cankers is stressed; optimal timing of spray applications is not enough. The model is used widely in California, where Zoller is in private practice.

The Maryblyt™ model (Steiner, 1990a, b; Lightner and Steiner, 1993; Steiner and Lightner, 2000; Fig. 15.1)

The Maryblyt™ model was developed in fire blight-prone apple orchards in Maryland, USA, where weather is often wet and warm. Its application was soon studied in other eastern states and, later, in other climatic areas. An important double advance was made with the development of this model. Firstly, Steiner determined the heat sum required before wetting for significant blossom infection to occur when flowers, colonized by the pathogen, were the source of inoculum. Secondly, he determined the heat sum required, following infection, for early signs of apple blossom blight to be seen. The model also considers blossom development rates, early canker activity, direct and indirect shoot blight and leafhopper activity. These risks have not been widely examined by others and are not discussed here.

Study of weather patterns indicated that the minimum conditions for blossom infection, in sequence, were: open, intact flowers; the accumulation of at least 110 DH above 18°C, a wetting event (rain \geq 0.25 mm, heavy dew or fog); a mean daily temperature of 15.6°C (this last value may be too high in cooler climates). Steiner used an adaptation of the DH approach used by Zoller. The DH sum is referred to as the relative epiphytic inoculum (or infection) potential. Warm days affect the rates of opening of new uncolonized flowers, rates of flower colonization and rates of spread of the pathogen between flowers by pollinating insects. The higher the DH sum, the higher the percentage of flowers containing the pathogen (Zoller, 1978; Zoller and Sisevich, 1979) and the

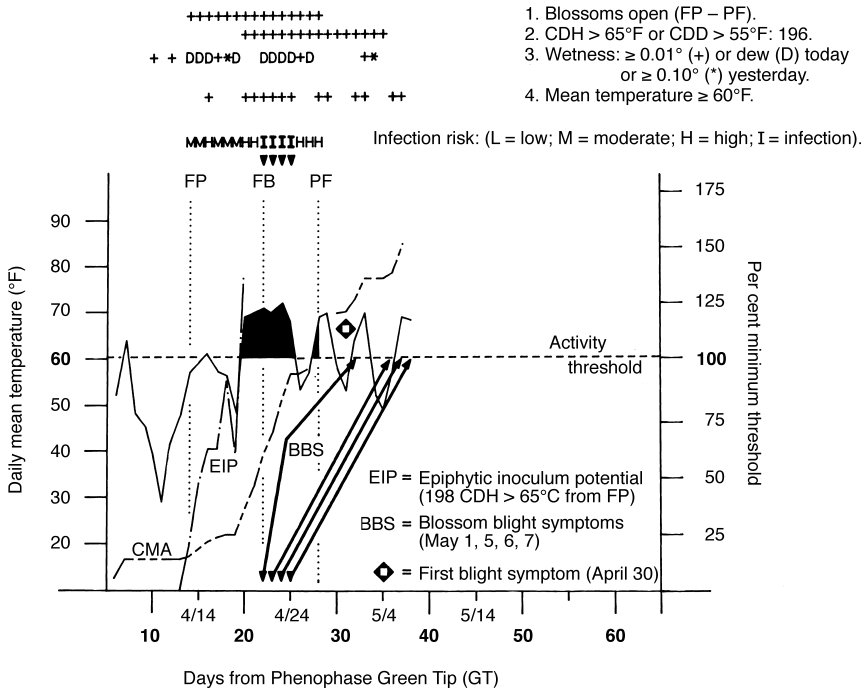


Fig. 15.1. A diagrammatic representation of the computer output of the Maryblyt™ model for West Virginia, 1985. There was a major outbreak of apple blossom blight then following high temperatures and dewfall during bloom. Early signs of blossom blight were seen within 1 day of the first predicted day. (The figure, slightly modified, is from van der Zwet and Beer, 1995.)

higher the inoculum levels in flowers are likely to be. This means that high disease incidence is often associated with high DH sums prior to a wetting event.

For estimating the time when early signs of apple blossom blight might be seen following infection, a DD sum above a mean temperature of 12.7°C is used. The critical sum for apple blossom blight was found to be about 50. Using the sine-wave function introduced for greater precision in cooler climates, this sum is now 57 (Lightner and Steiner, 1993). The precision of this simple heat sum for apple blossom blight is remarkable (Steiner, 1990a; Jones, 1992; van der Zwet *et al.*, 1994; Lightner *et al.*, 1999), but pear blossom symptoms sometimes appear earlier than expected (Gouk *et al.*, 1996; Moltmann, 1996). With Billing's integrated system (BIS) (see pp. 307–309), using DD sums above a mean temperature of 13°C, there is often high precision for apple blossom blight and, if the threshold sum is reduced, for other hosts and for shoot blight. The Maryblyt™ model allows for changes in threshold sums if symptoms are consistently seen early or late (Lightner and Steiner, 1993).

Since 1990, the Maryblyt™ model has been widely used in different countries and different climatic areas. There is agreement that it generally gives useful guidance on optimal timing of protective spray applications during bloom, especially if used with consideration of field risks (Jones, 1992; Bonn, 1993, 1996; Sobiczewski and Berczynski, 1993; van der Zwet *et al.*, 1994; Bazzi *et al.*, 1996; Gouk *et al.*, 1996; Moltmann, 1996). Full evaluation, however, is often hampered by low disease incidence.

Jones (1992) described some limitations of the original model and, some of these were endorsed by van der Zwet *et al.* (1994). Late flowers appearing after petal fall must be considered; allowance is now made for this (Lightner and Steiner, 1993). Wetting of flowers by dew or fog and the amount of damage caused by storms or other agents adequate to induce direct shoot blight are user judgements. Days during bloom without wetting when temperatures are equal to or above 26.6°C may be important (see also Mills, 1955; Billing, 1996). The model occasionally fails to indicate likely infection events and blossom blight is not always seen after such days. This last is a common complaint with other systems, but Jones (1992) suggested that seemingly false predictions are probably acceptable. With insignificant levels of blossom blight, there may remain enough inoculum for severe blight at a later date if protective sprays are not applied at all high-risk times during bloom.

For more detailed information on this comprehensive model, readers should consult the original papers. A Maryblyt™ computer program, with full details of the model as it is currently used, is available commercially. Methods for determining critical DH sums during bloom now take account of the life of individual flowers and the effects of spray applications (P.W. Steiner, personal communication).

Smith's fire blight model, Cougar blight (Smith, 1993, 1996, 1999a, b)

One advantage of the model developed by Smith for the semi-arid Washington State, north-western USA, is its ease of use. The model can be used in conjunction with regional weather data plus simple tabulated guides. If desired, it can be used in conjunction with an orchard weather monitor and a computer. Field risks, as well as weather-related risks, are considered and include: IP assessments from past and current disease observations (in or near the orchard or in the surrounding area); host susceptibility (cultivar, age, stage of growth, vigour); and blossom numbers. Another distinctive feature is the use of a 4-day heat sum prior to a wetting event.

In Washington State, weather is generally cool and dry during full bloom of both apples and pears. Widespread blossom blight is rare and post-bloom disease levels are usually low. Sporadic late flowers, after petal fall, can pose a problem on both hosts, but pears are normally worse affected than apples, because of secondary blossom in early summer. When the mean temperature line (Thomson *et al.*, 1982) or the Maryblyt™ model (Steiner, 1990a, b) were used, fire blight often failed to occur following postulated risks. Though a similar

problem occurred, Mills's (1955) DD approach and Zoller and Sisevich's (1978, 1979) DH model seemed to provide the best basis for a new approach tailored to the occurrence of fire blight in Washington State.

For weather-related risks, estimates of DH above 15.5°C are made for each 4-day period (not more) during bloom prior to wetting by rain or by 4 h or more of dew. To estimate DH from daily maximum and minimum temperature values, a simple conversion chart is available. This was based on *in vitro* growth rates of the pathogen (Schouten, 1987; see also Billing's revised system (BRS), pp. 305–306). The DH sum aims to provide an indication of possible pathogen populations in flowers. Weather forecasts are used to allow prediction of temperature and wetting risks. The threshold DH sum for determining low, medium and high risks of infection varies according to field risk estimates. The grower decides at which level of risk he/she wishes to apply protective sprays. In three high-disease-incidence years, those using the model applied protective agents in a far more cost-effective manner and had better disease control than those who did not (T. Smith, personal communication).

A logistic regression model (Beer *et al.*, 1984)

A tentative logistic regression model was developed in New York State, combining field and weather-related factors. The following risks were given scores: soil drainage and pH; cultivar; rootstock; tree age and growth characteristics; and previous disease records. Infection risks were judged using a modified form of the Billing's original system (BOS) approach. They placed emphasis on temperature on the day after rain (not on the day of rain or the day before). Further developments are not reported.

The development of systems and models in Europe

When fire blight was found in England in 1957, the use of streptomycin was not permitted and subsequent events showed that, for climatic reasons, it would rarely be cost-effective. Weather-related risk assessment, therefore, concentrated on infection risks during the whole growing season and optimal timing of searches following infection to ensure early surgical control measures. By 1990, fire blight was widespread in Europe and in Mediterranean areas and streptomycin was commonly used. Interest, therefore, turned to risk assessment systems developed in the USA. However, now that streptomycin resistance is a major problem, there is again concern to reduce risks at all times during the growing season and to control low levels of disease and minor reservoirs of inoculum.

Computerized systems developed in Europe used some features of BOS or BRS, so these systems are described first. Their precision might now be improved by following BIS methods, which incorporate some MaryblytTM principles.

Billing's original system, BOS (Billing, 1980a, b, 1984)

England was not the best climate for the development of risk assessment systems. Disease incidence is mostly very low, even on hawthorns (*Crataegus*), where it often remains uncontrolled. The development of BOS, and of BRS and BIS, which later superseded BOS, depended on published and unpublished reports of outbreaks in Continental Europe and the USA, as well as in England.

Potential daily doublings (PD) of the pathogen were estimated from *in vitro* growth rates (Billing, 1974). A tentative equation for calculating the theoretical incubation period (I-period) length (from infection to early symptoms) was based on a combination of PD sums and rain scores (Billing, 1976). Suggested graded IR days during bloom were based on daily temperatures and rainfall. Analyses were presented in a graphical form, showing IR days and with the subsequent I-periods as diagonal lines. It was suggested that the number and slopes of I-period lines was an indication of potential for fire blight activity (PFA) throughout the growing season, but this would not be true in all climatic areas.

Some users misunderstood and misused aspects of BOS. I-periods were thought to represent 'infection periods' (I-periods are now called disease development periods (D-periods)). High IR (which might relate only to one susceptible target close to a source of inoculum) were confused with high disease incidence risks. PFA judgements were sometimes based on I-period lines alone without reference to IR at the start of each I-period or to actual blossom periods at the time. The precision of the I-periods was sometimes overestimated. The tentative equation used for estimating I-period length in BOS, and later in BRS, was based on a mixture of stem invasion and blossom and shoot blight cases (Billing, 1976). This led to inclusion of a rain factor outside the main blossom period which, with hindsight, was not appropriate for direct infections.

Testers found that BOS usually reflected disease trends during bloom (when the rain factor was not used), but not always beyond. Infections occurred at fewer times than suggested (Billing and Meijneke, 1981; Paulin *et al.*, 1983; Meijneke and van Teylingen, 1984; Sobiskewski, 1984; Bonn, 1987).

Correlations are not always widely applicable. In England (1958–1968), using BOS, there was a correlation between the numbers of diseased pear and hawthorn trees and the numbers of completed I-periods or days with rainfall ≥ 2.5 mm between March and October (Billing, 1980a). This was valid then because, most disease was detected from July onwards, when stem invasion (favoured by high soil moisture) was likely. No such correlation would be expected in semi-arid areas or when blossom blight incidence is high. The correlations that Mills (1955) reported (see p. 299) between apple blossom blight severity and DD above 18.3°C with precipitation in New York State did not prove useful elsewhere.

Billing's revised system, BRS (Billing, 1990, 1992, 1999)

Weaknesses of BOS led to the development of BRS. The basic assumptions on which BRS was based are described fully by Billing (1992), where the importance

of taking account of field risks was emphasized. The main differences between BRS and BOS are: an improved set of PD values (Schouten, 1987); changes in the table of graded IR, notably the omission of all cool wet days and the inclusion of very warm, dry days (PD 11.0 or more) during bloom. The importance of the temperature on the day of rain or the day before (not after) was emphasized. The graphics were simplified by introducing symbols to indicate temperature and rainfall levels.

It was noted that rain at the end of a run of warm days often favoured blossom blight, especially when temperatures were in the range of 21–27°C (see 'Degree-days above 18.3°C' above) (Powell, 1965). D-period length estimates often gave useful (though rarely precise) guidance on the timing of searches, except in the case of apple blossom blight, where signs of disease took longer to develop following IR days. In spite of limitations, BRS gave useful guidance in climatic studies in Greece (Psallidas *et al.*, 1990).

Attempts were made to fit the Maryblyt™ model to the cases used to judge BRS precision without success, though the IR principles seemed useful in high-incidence blossom blight cases. For early signs of blight, the DD12.7°C mean sum of 50 was only suitable for apple blossom blight. Fortunately, solutions to these problems were found, which led to the development of BIS (see pp. 307–309).

Firescreens (Jaquart-Romon *et al.*, 1987; Jacquart-Romon and Paulin, 1991)

This computerized warning system was developed in France in the belief that an assessment of IP and the use of weather forecasts were essential features of a practical approach. An estimated IP was combined with a climatic potential (CP). The system assesses risks in the pre-bloom, blossom and post-bloom stages up to 31 July and takes account of secondary blossom and shoot growth periods.

When judging CP by weather analyses, some of BOS principles are used, including PD values, with emphasis on values of 9.0 or more for pre-bloom and blossom blight risks. With this value, maximum temperatures would usually be $\geq 21^{\circ}\text{C}$ and often higher and mean temperatures usually $\geq 15.5^{\circ}\text{C}$. For shoot blight risks, rain and storms are important factors. D-period estimates are used to judge times when fresh inoculum (IP) might be present. IP is judged by past and present disease, in the orchard or nearby, and by CP (D-period analysis). There is a scoring system for both CP and IP and weather forecasts are used to give advance warnings of risks. Using a combination of CP and IP risks, the computer program suggests times when: no action is needed; orchards should be visited for signs of blight and surgical control; protective sprays should be applied.

At two locations in France, Firescreens gave good guidance on spray timing in nine out of ten cases and an economy in the use of sprays was achieved (Lecomte *et al.*, 1996). In a 2-year study in Greece, Tsantios and Psallidas (1996) found that the use of the system saved two or three spray applications in orchards where no blight appeared. In a further pear orchard, the grower had not cut out any diseased parts and there was severe blight, in spite of strepto-

mycin spray applications. This re-emphasizes the importance of combining good orchard hygiene with protective spray applications.

A simulation model (Timmermans, 1990)

This model was developed from observations in Belgian pear orchards. It was described as a first step towards a system that integrated field and weather-related risks in a dynamic fashion. The need for adequate field records (including host phenology and storm records) and quantitative data on all aspects of fire blight epidemiology was emphasized. The tentative model included continual assessments of IP (based on past and current disease), inoculum spread risks due to insects and rain (especially during storms), host susceptibility and susceptible target numbers (flowers, young shoots and fruits) and storm or insect damage. The skill of the grower in removing diseased parts of trees would have an important effect on IP. Adaptations of BRS methods (Billing, 1990) were used when judging IR and disease development rates. The model reflected disease trends in current and past years.

Feuerbra and Anlafbra (Berger *et al.*, 1996)

These two mathematical models were developed in Germany for the assessment of regional and orchard risks. Perry pears and cider apples were at risk, as well as dessert pears and apples. The data input of both field and weather-related risks is supplemented by results of monitoring for the presence of the pathogen in flowers. For judging IR days, BRS methods (Billing, 1992) are followed. For D-period length estimates, the equation includes daily PD values and rainfall. Host susceptibility is included in both models. For the orchard-specific model, the phenological status of trees (including secondary blossom and shoot succulence) and susceptibility details for every cultivar are added. Tests in Germany indicate that Anlafbra reflects disease trends well.

Billing's integrated system, BIS (Billing, 1996; Berrie and Billing, 1997; Fig. 15.2, Table 15.2)

The corrected version of Billing's 1996 paper should be used as a reference.

In 1990, attempts were made to fit the MaryblytTM model to the cases used to judge BRS precision, but they were unsuccessful. The IR principles seemed useful when blossom blight incidence was high, but not when it was low.

In 1992, possible solutions were found to some problems. After some trial and error, all data were reanalysed according to the following assumptions: trace rainfall records are often an indication of heavy dew-fall; a DD sum of 17 above 18°C (DD18 max.) prior to a wetting event can replace the DH sum of 110 (though possibly with loss of precision); a DD sum above a 13°C mean value (DD13 mean) can replace the DD12.7 mean °C sum for judging D-period

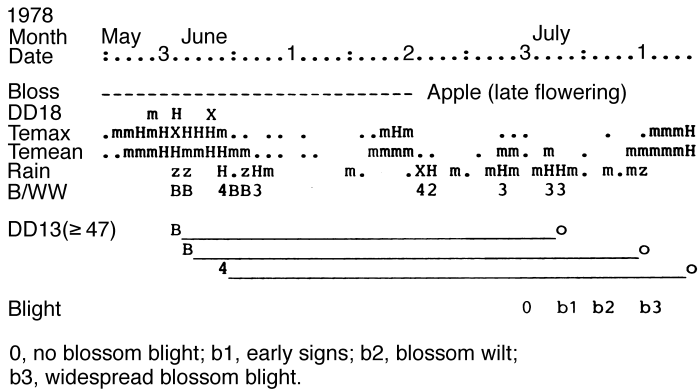


Fig. 15.2. BIS graphic for a late flowering apple orchard in south-west England, 1978. There was a major outbreak of apple blossom blight following unusually high temperatures and dewfall during bloom. Early signs of blossom blight were seen on the first predicted day.
Key to symbols: X, H, m, •: Extra, High, medium, low (see Table 15.2 for values); DD18, sum of degree days >18°C max; z, dew; B, blossom infection risk; WW, warmth/wetness score (infection risk); DD13, sum of degree days > 13°C mean; o, end of D-period, possibly with fresh ooze; b, blossom blight.

Table 15.2. Guide to symbols used in BIS graphics.

Symbol	Level	DD18 maximum (sum ≥)	Maximum temperature (°C ≥)	Mean temperature (°C ≥)	Rainfall (mm ≥)
U	Ultra	68	30	22	20
X	Extra	51	27	20	10
H	High	34	24	18	3
m	Medium	17	21	15	1
•	Low		18	13	<1
z					trace, dew

length, with a threshold sum of 47 for apple blossom blight and 17 for fire blight on all other hosts following direct infections of blossoms or shoots.

BIS combines the above modifications to the Maryblyt™ approach with improvements to BRS, which include: simplified graphics; a simplified table of IR scores (including days with high temperatures during bloom without recorded rain or dew); a clear distinction between direct infections and indirect infections, where disease progresses in bark from earlier blight (including overwintering stem and twig cankers), via the base of a shoot or blossom cluster. The importance of assessing field risks is again emphasized and rules for weather analyses are specified. Like BOS and BRS, BIS is intended for use

throughout the growing season. The main aim in the methodology is simplicity. Computer use is not essential but fire blight warnings (based on some BIS principles) are now incorporated in a computerized integrated warning system for apple diseases, ADEM (Xu and Butt, 1997).

The design of BIS is sufficiently flexible to allow for future evolution or adjustments to suit local conditions, including wet- and dry-bulb thermometer and leaf wetness records for mist and dew assessments, and for the conversion of daily temperature data to DH values. With the graduated IR table, BIS allows for low-incidence cases, where rain spreads inoculum locally from existing blight and overwintering cankers. Low disease incidence can provide important reservoirs of inoculum if risks become high later. Guidance on canker activity in spring is lacking at present.

The development and fine-tuning of BIS were based on more than 200 cases of fire blight in England, Continental Europe and the USA (published and unpublished reports). Of these, about 70 cases have allowed examination of the DD13 mean sums (using thresholds of 17 or 47) for precision when judging D-period length. Records of first signs of disease were not always available, but it seems that precision may often be close to that reported for the Maryblyt™ model for apple blossom blight. A recent study, using historical data from the experimental orchard in Dax, confirmed that the D-period length for pear blossom blight is shorter than that for most apple blossom blight (Lecomte *et al.*, 1998).

The graphics (Fig. 15.2, Table 15.2) have proved useful teaching tools, as well as giving a broad guide for experienced BIS users to fluctuations in risks through the whole growing season (the symbols used are easy to learn). Anomalous cases are easy to explore. Because the graphics are novel, non-users do not always appreciate the value of the graphics.

Use and applications of systems and models

Weather data and weather analyses

All too often published meteorological data are accepted at their face value, with a blind faith that is rarely justified by the facts

(L.P. Smith, cited by Coakley, 1985).

Weather records

Daily records may come from a variety of sources, including regional weather stations, automatic data loggers, thermographs and thermohygrographs and maximum and minimum thermometers and rain gauges in or near orchards. No record will be representative for all parts of all trees. The most important factor is that records should be regular, reliable and accurate and sufficiently representative for the location concerned. There should be no gaps in the daily records. All instruments need regular checks and maintenance. Thermograph

accuracy can be a problem (Thomson *et al.*, 1982). Data loggers can break down. Placement and proper protection are also important. Regional records from meteorological stations are often the most reliable; well-placed back up max./min. thermometers in or near orchards can provide useful checks on and major differences between regional and orchard records.

Conventions on recording times and reporting days vary. Midnight recording of temperature means that minimum temperature values may sometimes be before and sometimes after the maximum temperature record. With early morning records (07.00 to 09.00), the usual convention is that minimum temperature values come before maximum temperature values. With rainfall, midnight records mean that night rain following the daily maximum temperature may be split between two days, which is undesirable. When rainfall is recorded in the early morning, it is usual to report it for the previous day ('thrown back'). Where a system has precise rules for recording and reporting and for rounding of values, these should be followed.

Temperature and rainfall records

Errors introduced by temperature conversions and rounding are shown in Table 15.3. They are not great, but awareness of exact temperature values is important when comparing different systems. DD sums can prove unreliable in some climatic areas and DH sums are sometimes preferred. Where reliance is on daily maximum and minimum temperature values, tables based on sine-wave functions give useful guidance, as allowance is made for days when there are wide differences between maximum and minimum values. Temperatures above 32°C, the maximum temperature for growth of the pathogen, should be excluded.

Rainfall amounts can vary widely over short distances. Reliable mist and dew-fall records during bloom can be difficult to obtain (van der Zwet *et al.*, 1994) and direct observations of flower wetting are the ideal. If liquid is present in nectaries of flowers early in the morning, the %RH is probably high enough for infection (Parker *et al.*, 1956). Inspections before sunrise seem valuable, therefore, for any form of flower wetting, as well as for ooze detection. If rainfall records come from regional sources or orchard data loggers, a simple rain-gauge near the orchard provides useful backup. Trace rainfall records, leaf-wetness monitors or records of low vapour pressure deficits (VPD) can give indirect guidance on mist or dew possibilities. Precision of hygrographs is low, but wet- and dry-bulb thermometers give good guidance on VPD and %RH at higher levels.

The degree of tissue damage after storm and frost-damage risks is best assessed directly by observation but, records of thunderstorms, hail (usually very localized) and strong winds with gusts of 15 m s⁻¹ (35 m.p.h.) or more can give useful warning of the need to inspect trees. Frost damage is usually associated with temperatures of -2°C or less.

Weather forecasts now provide good guidance and can give a risk assessment system, normally based on past events, predictive value. They are now

Table 15.3. Important rounded °F and °C values used in risk assessments, including those showing discrepancies of 0.5°C or more. Degree-day (DD) and degree-hour (DH) conversions.

Maximum temperatures		Mean temperatures	
°F	°C (rounded)	°F	°C (rounded)
55	13	40 (4.4°C)	5 (41.0°F)
60 (15.6°C)	15 (59.0°F)	50	10
65	18	60 (15.6°C)	15 (59.0°F)
70	21		
75	24	57	14.0
80 (26.7°C)	27 (80.6°F)	58	14.5
85 (29.4°C)	30 (86.0°F)	60	15.5
90 (32.2°C)	33 (91.4°F)	62	16.5

DD and DH sums: °F to °C, subtract 32, multiply by 5/9 (0.56).

°C to °F, multiply by 9/5 (1.80) and add 32.

thought to be essential for optimal timing of protective spray applications. It is particularly important to learn in advance of the likelihood of unusually high temperatures during bloom, with risks of epidemic blossom blight.

Disease assessments and disease control

Disease incidence and severity

High levels of blossom blight are unusual even in blight-prone areas. The most important factor seems to be temperatures during bloom (not before or after), especially at or near full bloom, when target numbers are high (Zoller in van der Zwet *et al.*, 1988; Steiner, 1990a; Billing, 1992; van der Zwet *et al.*, 1994; E. Billing, unpublished records). There usually needs to be at least one wetting event after a warm period. Blossom blight incidence is often, but not invariably, related to the number of overwintering cankers (Zoller, in van der Zwet *et al.*, 1988; Smith, 1996). It is suspected that one active canker in or near an orchard can be enough to initiate early blight, which can later fuel epidemic blight if risks become high.

Broad assessments of between-host, season and/or area blossom blight risks are sometimes required. For comparing temperature levels and heat sums during bloom, actual flowering records are needed to allow for between-season differences in flowering. Failing these, methods used by pomologists to predict flowering times might give broad guidance.

Shoot and fruit blight incidence is often associated with the extent of any storm damage (especially hail damage) and the inoculum potential within the host trees or other hosts nearby, such as hawthorn. Wind-blown rain during

storms can spread inoculum widely. Cases involving insect attack are not well documented.

Tree damage can cause much more financial loss than crop losses. It is most common on pears and young apple trees, where stem invasion may be extensive. This is why early detection of blossom and shoot blight is important. Stem invasion rates are related to stem expansion rates and are likely to be favoured by high temperatures in conjunction with high soil moisture. Withholding irrigation can limit risks.

Disease control

All agents, including streptomycin, act best when applied before a wetting event favours infection. This means using weather forecasts to time applications. If many overwintering cankers are present, the effectiveness of a protective spray may be seriously diminished, especially on pears. There is a constant conflict between economy in the use of sprays and the need to protect early open flowers so that colonization and a few early infections do not fuel major disease later if risks become high. Some thresholds suggested for timing early sprays are summarized in Box 15.5 but their use is a matter for local judgement. So too are intervals between later sprays, where risk assessment systems give valuable guidance. The suggested thresholds may not be appropriate in all climatic areas. In the USA, problem areas include coastal areas in north-west California (Thomson *et al.*, 1982; Zoller, in van der Zwet *et al.*, 1988) and north-eastern states, including New York State (Steiner, 1990a; Billing, 1992). In the former, there are sometimes wide differences between daily maximum and minimum temperatures; in the latter, spring weather is often both wet and warm. The use of sine-wave functions or adjustments of thresholds may or may not go some way towards resolving these problems.

Routine inspection of trees is always important, but special searches for signs of new disease are necessary when high blossom or shoot blight risks are suspected. Early surgical measures will reduce levels of fresh inoculum and limit stem invasion risks. A fine judgement on timing is necessary to avoid costly, wasteful searches when infection risks have not been high or when symptoms are not sufficiently well developed for detection by inexperienced people. The use of DD sums above a mean temperature of 13°C (or 12.7°C) for judging optimal timing of searches has proved valuable, but between-host or cultivar differences regarding critical threshold sums need to be clarified.

Evaluating risk assessment systems and models

Fire blight is too sporadic to allow good comparisons of the validity and precision of the different systems in the near future. Underlying principles have been outlined here but, for a critical examination of what each has to offer, readers

Box 15.5. Some thresholds suggested for the timing of first spray applications during bloom depending on perceived risks.^a

Maximum temperature above 18.3°C if rain or high humidity is forecast (Mills, 1955)

Mean temperature line (16.7 to 14.4°C) has been crossed (Thomson *et al.*, 1982); or mean temperature 15.6°C or more (S.V. Thomson, Utah, personal communication)

Degree-hours above 18.3°C, 0.5 to 84 within 24h before rain is forecast (Zoller, in van der Zwet *et al.*, 1988)

Degree-hours above 18.3°C, forecast of 110 or more on a wetting day or a possible wetting day. Spraying before a lower risk threshold is sometimes justified (Steiner, 1990a)

^a Protective sprays are best applied before a wetting event. Perceived high risks include: young trees; highly susceptible trees; high overwintering canker risks; possibility of future warm weather and/or a wetting event. Protection of all trees may take 2 days.

must consult original papers. To avoid unrealistic expectations, system users must look at the evidence on which recommended methods are based; they are often tentative. The fact that systems are working hypotheses (not final solutions), which require further testing, is sometimes overlooked.

The choice of system will depend partly on whether or not protective sprays are used during bloom and whether a major concern is economy in spray use. For others, simple guidance that blossom infection is unlikely before temperatures during bloom reach 18°C (maximum) or 15°C (mean) and that risks may be high when maximum temperatures are often 21 to 27°C, with occasional dew or light rain, may suffice. A system developed in a semi-arid area may be less suitable in an area where weather is often wet and warm with storms. A system developed for apples may be less suitable for pears, and vice versa. Some people like to run two systems in parallel for some years. Other users will be more concerned with optimal timing of searches, but for this they will still need to identify IR days.

Users should be testers. They need to look immediately for possible reasons when a system seems to over- or underestimate risks, before vital evidence is lost. Reasons will usually be related to: IP; blossom or shoot susceptibility; target numbers (including late flowers); unusually hot, cold or wet weather; storm damage.

The question arises, how well can field risk factors be quantified? Some systems leave the judgement to the user; some suggest adjustments to critical weather risk thresholds for certain risks; some incorporate field risks in computer models. Ultimately, the user must follow carefully the intent and methods described by the designer and not push the system beyond its limits. If a user decides to make changes or to use only parts of a system, their approach becomes their responsibility and it should not be called by the name of the

system on which it was based (Billing, 1990). Further fine-tuning is likely with most systems, so users should consult authors for recent developments.

Conclusion

To ensure that a system or model is used to best advantage, users should consult the designer, who may have information on slight modifications or additional guidance on use. Risk assessment systems are not expert systems, but they are useful tools when trying to combat fire blight (Bonn, 1996; Deckers, 1996) provided that users do not have unrealistic expectations. Suggestions for future developments follow.

Better assessments are needed of overwintering canker risks in late summer and autumn, especially in relation to late flower and shoot infections, late storms exposing leaf traces on near-mature shoots and high soil moisture levels in warm weather encouraging late stem invasion. Postharvest copper sprays might be better targeted. More needs to be known about pre-bloom disease, especially factors affecting twig and stem canker extension and possible pre-bloom and early bloom infections, so that pre-bloom copper sprays could be better targeted.

With shoot blight, the role of sucking insects remains uncertain (Steiner, 1996). The roles of different insects in moving inoculum to first open flowers is also uncertain. Bees and flies may be important vectors for biological control agents (Nucló *et al.*, 1996; Vanneste, 1997), so perhaps factors affecting insect activity will now be an ongoing area of research.

For further progress, comparative tests on different systems and models need to be made whenever an opportunity arises. A pool of good test cases would be valuable.

Summary

Fire blight risk assessment systems and models have evolved in the light of new knowledge and deeper insight. In the USA, early simple approaches are now replaced by those which consider the colonization of open flowers by the pathogen. The MaryblytTM model was a notable advance, as it described concisely high apple blossom infection risks and also the likely rate of disease development following infection, using a simple DD or DH sum above a mean temperature of 12.7°C. The importance of overwintering cankers in spring is emphasized in most approaches. In Europe, systems and models often used the concept of potential daily doublings of the pathogen (based on daily temperature values), plus daily rainfall and storm records, in conjunction with field risks. Latterly, features of the MaryblytTM model were incorporated in an integrated system (BIS), which also retains elements of earlier European systems. The precision of estimates of disease development rates can be remarkably high.

Agreement between the Maryblyt™ model and BIS is often good for apple blossom blight. Different views on critical DD sums for blossom blight on other hosts and for shoot blight need to be resolved. For good risk assessments, all approaches need reliable field records, as well as reliable weather data. When weather-related risks and inoculum levels are both high, protective agent applications may prove inadequate, however well tuned.

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Notes added in proof

Since this chapter was written, the *Proceedings of the Eighth International Workshop on Fire Blight* have been published (Momol and Saygili, 1999). There is now further guidance on the principles and use of Smith's model (Cougarblight 98C) and of BIS. A 15-year summary evaluation of the Maryblyt™ model on apple in West Virginia is presented. Other contributions include useful comments on the application of different models and systems in different European countries but, so far, too few seasons have been studied to judge the relative merits of different approaches. The actual value of using a sine curve to determine hourly temperatures from daily maximum and minimum temperatures in relation to the precision of risk assessment needs further study in a variety of climatic areas. Sine wave functions should not be used for BIS degree day sums. Recently, a revised version of the Maryblyt™ model has become available (Steiner and Lightner, 2000).

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Biological Control of Fire Blight

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Introduction

Over the last 20 years, biological control of fire blight has advanced from a subject of basic research to a feasible component of an integrated disease management programme. This advance has been propelled by selection of effective antagonist strains, by enhanced knowledge of the mechanisms by which these strains suppress disease and by increased understanding of the ecology of bacterial epiphytes on plant surfaces. Moreover, orchardists have begun to accept biological control as a complementary strategy, which can be used effectively with other forms of disease suppression.

Impetus for the advancement and implementation of biocontrol of fire blight has resulted from several factors, including the increasing importance of the disease, the development of resistance in *Erwinia amylovora* to the antibiotic streptomycin and societal desires to enhance the safety and sustainability of agricultural production systems. As discussed in Chapter 3, *E. amylovora* continues to spread to new countries, many of which do not allow the use of antibiotics for disease suppression. In some countries with the pathogen (e.g. the USA), the spectrum of commercial apple cultivars has expanded and shifted towards those with greater susceptibility to fire blight (Thomas and Jones, 1992). Also in the USA, resistance in *E. amylovora* to streptomycin (Moller *et al.*, 1981; Loper *et al.*, 1991; McManus and Jones, 1994; Stockwell *et al.*, 1996b; Jones and Schnabel, Chapter 12) has accentuated the need for alternative control strategies. To be viable, these alternative strategies need to mesh with consumer and food safety standards, which, in many countries, have created a marketing and regulatory climate that

requires growers to produce high-quality fruit with minimal residues of synthetic chemicals.

Research on the biological control of fire blight has been focused on the interaction between an antagonist, usually bacterial, and *E. amylovora* on stigmatic and hypanthial surfaces of pear or apple blossoms (Vanneste, 1996). An effective interaction prevents floral infection by *E. amylovora*, and therefore prevents the direct damage (i.e. loss of yield and fruit-bearing surface) that results from infection. More broadly, suppression of floral infections reduces the inoculum of *E. amylovora* available for other phases and cycles of the disease, including shoot infections during the same season and floral infections in the following season. Therefore, biological control, like chemical control, should be valued both for its direct contribution to crop protection and for the indirect effects this protection has on maintaining pathogen populations at manageable levels in the orchard.

Our goal in this chapter is to review the work that has brought biological control of fire blight from research labs to practical use in commercial pear and apple orchards. The discussion emphasizes biological interactions occurring at the population (orchard) level, and is biased towards antagonist strains that are commercially available or are nearing commercial availability in the USA. These strains include *Pseudomonas fluorescens* strain A506 (PfA506) and *Erwinia herbicola* strain C9-1 (EhC9-1) (synonym: *Pantoea agglomerans* strain C9-1), which are representative of the two genera of Gram-negative bacteria that have been investigated in the overwhelming majority of the published research on biological control of fire blight. We note, however, that researchers continue to evaluate new antagonists for the control of *E. amylovora*, such as non-virulent strains of *E. amylovora* (Tharaud *et al.*, 1997), yeasts, Gram-positive bacteria and mixtures of bacteriophages specific to *E. amylovora* (Ritchie and Klos, 1977; Palmer *et al.*, 1997). Although these agents have shown promise in cultural tests or greenhouse assays, they have not been tested under field conditions to the same extent as *E. herbicola* and *P. fluorescens*. With further development, we expect that new, efficacious antagonists could be readily integrated into current biologically based methods of fire blight management.

Biology of fire blight suppression by bacterial antagonists

The stigmatic surface: the site of biological control

The surface of the stigma, located on top of the floral pistil, is the site where bacterial biocontrol agents must interact with and successfully antagonize *E. amylovora* (Hattingh *et al.*, 1986; Thomson, 1986; Wilson *et al.*, 1989; Vanneste, 1995). Stigmatic surfaces are studded with epidermal papillae, between which are large intercellular spaces where bacteria can reside. Cells located at the base of the papillae secrete nutrient-rich exudates that bacteria within the intercellular spaces can utilize for growth (Wilson *et al.*, 1989). As a

blossom opens, the stigmas are generally devoid of bacteria (McLaughlin *et al.*, 1992). With time, the exposed stigmas are colonized and high populations of bacterial epiphytes, including *E. amylovora*, develop on the surfaces. Large, epiphytic populations of *E. amylovora* on stigmas are important for two reasons: first, to increase the probability of successful infection of a flower (Hirano and Upper, 1983; Johnson *et al.*, 1993b) and, secondly, to increase the likelihood that the pathogen will be spread from a colonized blossom to a non-colonized blossom by rain (van der Zwet and Keil, 1979) or a pollinating insect, honeybees in particular (Pierstorff and Lamb, 1934; Hildebrand and Phillips, 1936; Kiett and Ivanoff, 1941; Van Laere *et al.*, 1981). Most evidence indicates that *E. amylovora* does not infect a stigma or floral style directly, but instead gains entry into the host through natural openings (nectarthodes) located in the floral cup at the base of the pistil (Thomson, 1986; Wilson *et al.*, 1989; Vanneste, 1995). Infection through nectarthodes occurs principally from the washing of pathogen cells from the stigma into the floral cup by rain or heavy dew. Development of epiphytic populations of *E. amylovora* on stigmas is influenced greatly by temperature (Thomson *et al.*, 1982). During warm weather, the pathogen's ability to multiply rapidly, combined with intense insect activity, can result in an epiphytic population of the pathogen developing in a large proportion of blossoms over a short period of time (Thomson *et al.*, 1975; Nuclo *et al.*, 1998).

Biological control of fire blight occurs when a bacterial antagonist establishes and develops a large population on the stigmatic surface prior to the establishment of *E. amylovora* (Wilson *et al.*, 1992; Johnson *et al.*, 1993b; Wilson and Lindow, 1993). These populations, through a combination of mechanisms, then suppress the establishment and epiphytic growth of the pathogen. Suppression of the increase in population size of *E. amylovora* on stigmatic surfaces reduces the probability of floral infection and spread of the pathogen to other blossoms. Effective biological control requires that most stigmatic surfaces in the orchard are colonized by the bacterial antagonists (Johnson *et al.*, 1993b; Lindow *et al.*, 1996), and that the population of the antagonist on these surfaces is large. A sufficiently large antagonist population is usually in the range of 10^5 – 10^6 colony-forming units (cfu) of the bacterium per flower, for which the latter number is considered the upper limit (carrying capacity) of population size for bacterial epiphytes on an apple or pear blossom (Wilson *et al.*, 1992; Wilson and Lindow, 1993). Fire blight is a good candidate for biological control because the bacterial antagonists need to persist on the nutrient-rich, stigmatic surfaces for only about a week to suppress blossom infection effectively.

Bacterial antagonists of *E. amylovora*

The bacterial species *P. fluorescens* and *E. herbicola* have been investigated widely for their ability to suppress floral infection by *E. amylovora* (Vanneste, 1996). PfA506 has been available commercially for fire blight suppression since 1996

(BlightBan A506™, Plant Health Technologies, Boise, Idaho). This bacterium, first isolated from pear in California (Lindow, 1984), has decreased the incidence of fire blight in plots located in California (Lindow *et al.*, 1996), Oregon and Washington (Johnson *et al.*, 1993b; V.O. Stockwell, unpublished data). *PfA506* can also suppress the severity of frost injury caused by ice nucleation-active strains of *Pseudomonas syringae* (Lindow *et al.*, 1996) and lenticel russetting of pear fruits caused by other bacterial epiphytes (Lindow, 1987). *PfA506* is a good colonizer of pear and apple stigmas in conditions that are typical during early to mid-bloom of pome fruits in the Pacific Northwest region of the USA (Johnson *et al.*, 1993b; Stockwell, *et al.*, 1996a). Generally, the populations established by *PfA506* on blossoms are large (10^5 – 10^6 cfu per blossom) under field conditions; however, only 50–70% of the treated blossoms may have detectable populations of *PfA506* (Stockwell *et al.*, 1992, 1998). The percentage of blossoms colonized by *PfA506* correlates inversely with the percentage of diseased blossoms (Johnson *et al.*, 1993b; Nuclo *et al.*, 1998). Thus, while the majority of the blossoms are commonly 'protected', a sizeable minority are not colonized by the antagonist and may be vulnerable to infection by *E. amylovora*. On average, a 40–60% reduction in incidence of fire blight on blossoms has been achieved with applications of *PfA506* in plots in the Pacific Northwest (Johnson *et al.*, 1993b; V.O. Stockwell, unpublished data) and in plots in California during the past 16 years (Lindow *et al.*, 1996). The efficacy of *PfA506* approached or equalled that obtained with chemical control in many of these field trials.

A second antagonist, *EhC9-1*, isolated from apple in Michigan (Ishimaru *et al.*, 1988), has been an effective agent for the control of fire blight in plots located in Oregon and Washington (McLaughlin and Roberts, 1992; Johnson *et al.*, 1993b; Stockwell *et al.*, 1996a). Like *PfA506*, *EhC9-1* is an excellent colonizer of stigmatic surfaces of pear and apple. An effort to commercialize *EhC9-1* for control of fire blight on pome fruits has been initiated and an application for an experimental use permit for *EhC9-1S* (a spontaneous streptomycin-resistant mutant of *EhC9-1*) was submitted to the US Environmental Protection Agency in the spring of 1997 (S. Kelly, Plant Health Technologies, Boise, Idaho, personal communication). Treatment with *EhC9-1* (or *EhC9-1S*) has decreased the incidence of fire blight between 50 and 80% compared with the incidence on water-treated blossoms (Johnson *et al.*, 1993b; V.O. Stockwell, unpublished data). The efficacy of biological control by *EhC9-1* has approached or equalled that obtained with streptomycin and usually exceeds the level of control provided by *PfA506*. In field plots, the populations established by *EhC9-1* on blossoms average 10^4 – 10^6 cfu per blossom (Stockwell *et al.*, 1992, 1996a, 1998; Johnson *et al.*, 1993b). Similarly to findings with *PfA506*, commonly 40–70% of the treated blossoms have detectable populations of *EhC9-1* (Stockwell *et al.*, 1992, 1998).

Several other strains of *E. herbicola* have shown promise for suppression of blossom infection by *E. amylovora*. These strains include strains *Eh252* (Vanneste *et al.*, 1992), *Eh318* (Wright and Beer, 1996), *Eh112Y* (Wodzinski *et al.*, 1994), *Eh1087* (Kearns and Hale, 1996), *EhH19N13* (Wilson *et al.*, 1990)

and Eh325 (Pusey, 1997). Like EhC9-1, the relative effectiveness of each these strains is apparently correlated with the production of antibiotics that are inhibitory to the pathogen. Additional discussion of these strains can be found in Vanneste (1996).

Mechanisms of biological control by bacterial antagonists

Production of antibiotics and site and nutrient competition are considered to be the principal mechanisms used by *E. herbicola* and *P. fluorescens* to antagonize the fire blight pathogen (Wilson and Lindow, 1993; Vanneste, 1996). EhC9-1 produces two distinct antibiotics (putative β -lactams) called herbicolin O and I (Ishimaru *et al.*, 1988). Of 55 isolates of *E. amylovora* recovered from commercial orchards in the Pacific Northwest region of the USA, all were sensitive to herbicolin O in culture assays and 53 were sensitive to herbicolin I (V.O. Stockwell, unpublished data). Herbicolin O inhibits the growth of a broad range of bacterial genera, whereas the toxicity of herbicolin I is more specific, inhibiting the growth of *E. amylovora*, *Bacillus cereus* and *Staphylococcus aureus* (Ishimaru *et al.*, 1988). The importance of antibiotic production by EhC9-1 in the suppression of field populations of *E. amylovora* has not been evaluated directly, but correlative evidence indicates that herbicolin production contributes to the effectiveness of this strain. Herbicolin O and I isolated from EhC9-1 decreased the development of fire blight symptoms on immature pear fruit inoculated with a strain of *E. amylovora* that was sensitive to both compounds. In contrast, EhC9-1 only partially reduced the severity of symptoms on pear fruit caused by a herbicolin-insensitive mutant of *E. amylovora* (Ishimaru *et al.*, 1988). Similarly, Eh252-10:12, a Tn5-mutant of Eh252 that no longer produces its antibiotic (a putative microcin), did not reduce symptom severity on immature pear fruit as well as its wild-type parent (Vanneste *et al.*, 1992). For both EhC9-1 and Eh252-10:12, however, partial suppression of disease under conditions of muted antibiotic activity is an indication that site and nutrient competition also contributes to the overall effectiveness of these strains.

In contrast, antibiotics inhibitory to the growth of *E. amylovora* have not been detected in cultures of *P. fluorescens* A506 (Wilson and Lindow, 1993). This bacterium apparently suppresses the pathogen by competing for sites and nutrients required for the growth of *E. amylovora*. In greenhouse studies, Wilson and Lindow (1993) found that stigmatic populations of *E. amylovora* were maintained below 10^5 cfu per blossom when PfA506 was inoculated on to pear blossom 72 h in advance of the pathogen; however, co-inoculation of PfA506 and *E. amylovora* did not result in suppression of the population size of the pathogen. Conversely, in a similar experiment (Wilson *et al.*, 1992), a herbicolin-producing strain of *E. herbicola* co-inoculated with *E. amylovora* suppressed both the growth rate and population size of the pathogen. Because PfA506 attained a population size that usually exceeded 10^6 cfu per blossom at 70–80 h after inoculation, Wilson and Lindow (1993) concluded that the ability of this antagonist

to suppress the pathogen when inoculated 72 h in advance was due to the pre-emptive sequestration of mutually required growth-limiting resources. *PfA 506* also colonizes the nectaries of pear blossoms, where resource sequestration by the antagonist may further reduce the probability of successful infection by *E. amylovora* (Wilson and Lindow, 1993).

Experimental evaluation of biological control

Laboratory-based screening for antagonist efficacy

Many studies designed to screen putative antagonists and investigate the mechanisms of biological control of fire blight have employed media-based and immature pear fruit assays to measure inhibition of *E. amylovora* by antagonist strains (Wrather *et al.*, 1973; Beer and Rundle, 1983; Isenbeck and Schultz, 1985; Ishimaru *et al.*, 1988; Nicholson *et al.*, 1990; Vanneste *et al.*, 1992; Kearns and Mahanty, 1993). Fruit- and media-based assays have been useful for investigating the effects of antibiotic production by *E. herbicola*, or for conducting a preliminary screen of the efficacy of antagonist strains for which antibiotic production is considered the principal mode of action (a review of these methods was published by Vanneste (1996)). Nonetheless, Wilson and co-workers (1990, 1992) found that laboratory-based assays with fruit did not always correlate with biocontrol effectiveness on blossoms. Moreover, antagonists that grow rapidly and outcompete the pathogen for essential resources on blossoms, e.g. *PfA 506*, often perform poorly in media and immature pear fruit assays (Mercier and Lindow, 1996). Most researchers now view measurement of the suppression of pathogen populations on blossoms as a superior approach to identifying effective antagonist strains (Andrews, 1985; Mercier and Lindow, 1996; Pusey, 1997).

To facilitate the use of flowers in antagonist screening, Pusey (1997) developed methods to increase the non-seasonal availability of pomaceous blossoms. Crab-apples were selected as the source of flowers, because of their high flower productivity on current-season growth, high susceptibility to fire blight and availability from nurseries. Potted crab-apple blooms after transfer from a cold room to a greenhouse or after removing all leaves and treating the buds with 1% cytokinin and 0.1% gibberellin, which eliminates the need for cold dormancy. To use crab-apple blossoms for screening, peduncles of detached blossoms were placed into vials containing a sucrose (10–25%) and maintained in a growth chamber (24°C), where the blossoms supported growth of epiphytic bacteria (Pusey, 1997). Small droplets of a suspension of an individual antagonist strain (10^8 cfu ml⁻¹) were pipetted on to the stigmas of each blossom, and this was followed after 24 h with droplets containing *E. amylovora* (10^6 cfu ml⁻¹). In some trials, population sizes of the antagonists and pathogen on inoculated blossoms were monitored for up to 4 days; in other trials, blossoms were misted at 24–48 h after introduction of the pathogen and then observed for fire

blight symptoms after 7–8 days. In evaluating this technology, Pusey (1997) found that both the herbicolin-producing *EhC9-1* and the non-antibiotic-producing *PfA 506* were excellent colonists of crab-apple flowers, and that both grouped among the better strains for their ability to suppress growth of the pathogen.

Mercier and Lindow (1996) also addressed the problem of efficiently identifying effective bacterial antagonists of *E. amylovora* on blossom surfaces. In their study, *E. amylovora* was transformed with the *iceC* gene from *P. syringae*, which encodes for a protein that nucleates ice formation. Over the temperature range of -2 to -4°C , Mercier and Lindow (1996) found that a blossom super-cooled in water and colonized epiphytically by the Ice^+ strain of *E. amylovora* froze at a temperature that was correlated positively with the logarithm of the pathogen population size. Blossoms colonized by bacterial antagonists that effectively suppressed growth of the pathogen were identified as those having the smallest incidence of freezing at the temperature that froze 95% of blossoms inoculated with Ice^+ *E. amylovora* only. Mercier and Lindow (1996) concluded that estimation of *E. amylovora* populations based on the freezing temperature of blossoms is simpler and less expensive than the use of dilution plating for the same purpose. Identification of effective antagonists based on the freezing method, however, requires a relatively large number of replications.

Evaluation of fire blight biocontrol in orchards

The activities of bacterial antagonists in a laboratory or greenhouse are not always a good predictor of their behaviour under field conditions. For example, greenhouse- and growth-chamber-grown plants often support greater populations of epiphytic bacteria than plants grown under field conditions (O'Brien and Lindow, 1989; Loper and Lindow, 1993; Beattie and Lindow, 1994). Furthermore, bacterial colonization of plant surfaces and the production of secondary antimicrobial metabolites in field environments are influenced by many factors that may not be reproduced in greenhouse or growth-chamber experiments, including differences in the physiology and growth of plants, environmental constraints imposed by variable weather patterns and the influx of competing, indigenous microorganisms. Consequently, most recent field studies that have investigated biocontrol of fire blight intensively have measured bacterial populations on blossoms in addition to measurement of disease response (Johnson *et al.*, 1993b; Lindow *et al.*, 1996; Stockwell *et al.*, 1996a; Nuclo *et al.*, 1998). Measurements of bacterial population dynamics provide comparative data on colonization of blossoms by individual strains, the effect of fluctuating environments on antagonist growth, the magnitude of pathogen suppression and an insight into the design of experiments and inoculation protocols.

In the field, sizes of bacterial populations, including those of *E. amylovora*, are usually estimated by dilution-plating the wash from individual blossoms, although techniques such as the rubbing of floral stigmas on culture media (i.e.

stigma streaking (Thomson, 1992)) can provide data as to whether or not a bacterial strain is present on a blossom. Population estimation by dilution-plating or stigma streaking is facilitated by the use of a culture medium that is selective for the growth of *E. amylovora* (Miller and Schroth, 1972; Ishimaru and Klos, 1984) or the antagonist strains, and often by the use of antibiotic-resistant (e.g. rifampicin, nalidixic acid) bacterial strains (Lindow *et al.*, 1996; Nuclo *et al.*, 1998). Because bacterial populations can be highly variable among field-sampled blossoms, suitable replication of blossom samples and experimental units is an important consideration. Detection of the suppression of *E. amylovora* populations, for example, may require four to seven replications of each treatment (arranged in a randomized block design) with eight to 32 blossoms sampled from each experimental unit on each of several dates (Johnson *et al.*, 1993b; Lindow *et al.*, 1996; Mercier and Lindow, 1996).

Based on dilution-plating, Johnson *et al.* (1993b) found that the early establishment of large populations of PfA506 and EhC9-1 on pear blossoms suppressed both the number of pear blossoms on which honeybee-dispersed *E. amylovora* was detected and the size of established pathogen populations. The effects of the antagonists on pathogen establishment and population size were consistent with the observed suppression of disease. Incidence of diseased blossom clusters was most strongly correlated with the proportion of blossoms that developed high populations of the pathogen ($> 10^5$ cfu per blossom), but was only correlated weakly with the proportion of blossoms that supported *E. amylovora* populations of any size. Nuclo *et al.* (1998) came to a similar conclusion in studies concerned with the natural spread of PfA506, EhC9-1 and *E. amylovora* among pear blossoms. In this study, both PfA506 and EhC9-1 moved gradually from a row of trees that had been treated with a mixture of these antagonists to non-treated blossoms located up to four tree rows from the treated row. Measured gradients of the incidence of blossoms with *E. amylovora* populations greater than 1×10^5 cfu per blossom and of the incidence of diseased blossom clusters increased significantly with distance from the row of trees treated with the antagonist mixture. The gradients describing the incidence of large pathogen populations and disease were inversely related to gradients that described the spread of PfA506 and EhC9-1 to non-treated trees (Nuclo *et al.*, 1998). Gradients were not observed, however, for the incidence of blossoms with detectable pathogen populations of any size.

In the above studies (Johnson *et al.*, 1993b; Nuclo *et al.*, 1998), honeybees were employed to disperse freeze-dried cells of *E. amylovora* to the blossoms of experimental trees, which were located inside a large shade-cloth enclosure. The reason for infesting honeybees with the pathogen was to mimic the method by which *E. amylovora* is introduced to blossoms under natural conditions. In evaluating fire blight biocontrol, the method used to introduce the pathogen on to experimental trees can dramatically affect the observed efficacy of introduced antagonist strains. In particular, the widely used inoculation method of spraying aqueous suspensions of *E. amylovora* can result in such severe infection that proven antagonists (and chemicals) for control of fire blight appear ineffective

(McLaughlin and Roberts, 1992). One reason for the overwhelming disease severity associated with spray inoculation of *E. amylovora* may be that the dose of the pathogen deposited on to floral surfaces, in particular the nectary, is large enough to cause infection directly, and the natural biology, in which *E. amylovora* attains an effective population size (dose) by first growing epiphytically on stigmatic surfaces, is bypassed by the inoculation method. Lindow *et al.* (1996) also concluded that antagonists of *E. amylovora* are more effective when used in orchards where disease is incited by indigenous populations of *E. amylovora*. In trials conducted over several years, PfA506 reduced incidence of fire blight caused by natural pathogen populations by 70% (Lindow *et al.*, 1996), compared with the 30–60% reductions that are observed typically in spray-inoculated trials (V.O. Stockwell and K.B. Johnson, unpublished data). The mean incidence of natural infection in the experiments by Lindow's group was estimated to be 0.05% of blossoms, which is about 100- to 1000-fold smaller than the typical incidence of a diseased blossom in a spray-inoculated trial. The low incidence of natural infection required plot sizes of 25 to 200 trees in each of four replications to ensure enough disease to measure meaningful differences among individual treatments. Spray-inoculated trials typically have only one tree per replication within a block.

Population dynamics of antagonist mixtures

Individual antagonists introduced into orchards are effective agents for fire blight suppression; however, for fire blight (and for the biological control of plant diseases in general), the commercialization of individual antagonists to suppress disease has been limited somewhat by inconsistencies in the level of control obtained from location to location and from year to year. These inconsistencies are often attributed to inadequate establishment or growth of antagonists, owing to fluctuating or non-uniform environmental conditions. The use of mixtures of antagonist strains has been proposed as a strategy to reduce these inconsistencies and is being currently investigated in several laboratories (Stockwell *et al.*, 1992; Vanneste and Yu, 1996; Nuclo *et al.*, 1998). In the design of antagonist mixtures, individual components are chosen ideally to maximize the following criteria: diversity in mechanism of biocontrol, total antagonist population size, breadth of the nutritional niche utilized by antagonist strains, and growth response of antagonist strains over a broad range of environmental stimuli. Fulfilment of these criteria is thought to increase the odds of establishing a robust and stable 'community' of antagonists on plant surfaces and, if successful, this stability provides the mixture with a potential advantage in disease suppression over individual antagonist strains.

Based on these criteria, a mixture of the species *P. fluorescens* and *E. herbicola* has shown potential as a strategy to improve the consistency of fire blight biological control (Stockwell *et al.*, 1992; Vanneste and Yu, 1996; Nuclo, 1997; Nuclo *et al.*, 1998). PfA506 and EhC9-1 have complementary mechanisms of

biocontrol (Ishimaru *et al.*, 1988; Wilson and Lindow, 1993), and they differ in maximal temperatures for growth in culture (27°C for *PfA506* and 37°C for *EhC9-1*), tolerance to desiccation stress and UV radiation (V.O. Stockwell, unpublished data) and ability to utilize various carbon and nitrogen sources (Wilson and Lindow, 1994). Individually, both antagonists colonize pear and apple blossoms, but the proportion of blossoms that each bacterium colonizes varies from year to year. For example, in years that had limited rainfall and warm daytime temperatures (16–22°C) during bloom, *PfA506* was recovered from only 20–40% of sampled blossoms, whereas *EhC9-1* was established on 70–80% of the blossoms. In contrast, *PfA506* was recovered from the majority of the blossoms sampled in field trials where rains were frequent and daytime temperatures were lower (10–12°C). When applied as a mixture, the incidence of establishment of either *PfA506* or *EhC9-1* on pear blossoms has averaged 80–90% of treated blossoms (Stockwell *et al.*, 1992; V.O. Stockwell, unpublished data). Similarly, in competition experiments, in which the proportion of applied cells of *PfA506* and *EhC9-1* were varied while total cell density was kept constant, the mean population size of both antagonist strains on pear blossoms was typically greater than expected, according to de Wit replacement series analysis (Nuclio, 1997). These attributes of antagonist mixtures, i.e. larger populations established on a higher proportion of blossoms, are important, because each blossom on a tree is a potential infection site for the pathogen.

Nonetheless, even though *PfA506* and *EhC9-1* have established higher combined populations on a greater proportion of blossoms when applied as a mixed inoculum, the control efficacy of the mixed antagonists in plots inoculated with *E. amylovora* is not usually better than that of a single antagonist applied alone (V.O. Stockwell, unpublished data). Vanneste and Yu (1996) obtained similar results in field experiments in New Zealand, where *PfA506* was applied as a mixed inoculum with *E. herbicola* 252. The lack of additive or synergistic control from the application of a mixture of *PfA506* and strains of *E. herbicola* is unexpected, given the current understanding of the importance of incidence of antagonist establishment and population size of antagonists in suppression of *E. amylovora*. This unexplained difference between expected disease suppression based on population dynamics of antagonist mixtures and observed disease suppression is in need of further research.

Strategies for implementation of biological control

Delivery and establishment of bacterial antagonists in blossoms

Establishment of populations of bacterial antagonists in blossoms is the most critical step in implementing biological control of fire blight in commercial orchards. Once established, there is ample evidence that populations of bacterial antagonists in orchards are relatively resilient (Lindow *et al.*, 1996; Stockwell *et al.*, 1996a), and that they can become partially self-sustaining by spreading

naturally from blossom to blossom (Nuclo *et al.*, 1998). The initial process of establishment, however, appears to be more variable, and potentially influenced by the method of inoculum preparation, the method of application, insect activity, bloom stage and orchard temperatures.

Stockwell *et al.* (1998) demonstrated that inoculum consisting of cells of *PfA506* and *EhC9-1* that had been freeze-dried and resuspended in water established epiphytic populations on blossoms more consistently (large populations on a greater proportion of flowers) than suspensions of cells prepared from cultures growing actively on artificial media. In this study, bacterial antagonists were sprayed in the orchard at midday, when blossoms would be exposed to UV radiation and rapid drying. Differences in establishment between freeze-dried cells and fresh cells from solid media were evident at the time of the first blossom sample, 4 h after treatment. Over five experiments, lyophilized cells of *PfA506* and *EhC9-1* became established in 70–100% of treated blossoms. Fresh cells, in contrast, established very poorly in several experiments, particularly when the plant surface dried quickly after application. The process of freeze-drying exposes bacterial cells to stresses imposed by freezing and desiccation, and apparently this process selects for a subset of cells better able to survive stresses encountered immediately upon application to plant surfaces (Stockwell *et al.*, 1998). Commercial formulations of *PfA506* and *EhC9-1* are prepared by freeze-drying, but this step is not always taken in research experiments. Establishment of *E. amylovora* in blossoms was also improved by freezing-drying the bacterial cells prior to application in the field (Stockwell *et al.*, 1998). This improvement in consistent establishment of *E. amylovora* allows inoculum suspensions to be prepared at lower cell concentrations, which increases the likelihood that the relative efficacy of applied treatments will reflect the responses that would have occurred under conditions of natural infection.

Establishment of bacterial antagonists of *E. amylovora* in pear and apple blossoms via honeybee vectors has been proposed as a method for implementation of biological control in commercial orchards (Thomson *et al.*, 1992; Vanneste, 1996). To use bees for this purpose, a device termed a 'pollen insert', which contains bacterial inoculum, is attached to the entry platform of a beehive. Bees become contaminated with a preparation of the bacterial antagonists as they exit the hive through the pollen insert; the bacteria are then transferred to floral surfaces as the bees forage for pollen and nectar. Inoculum preparations placed into the insert are either a dust of freeze-dried bacterial cells (Johnson *et al.*, 1993a) or pollen that has been soaked in a suspension of the antagonist strain and then dried (Thomson *et al.*, 1992). *PfA506*, *Eh318* and *E. amylovora* vectored in this manner have become established on stigmatic surfaces of apple or pear (Thomson *et al.*, 1992; Johnson *et al.*, 1993a). Johnson *et al.* (1993a), however, examined the efficiency of honeybees as vectors of bacteria and concluded that the insects were probably not as efficient as orchard sprayers for primary establishment of bacterial antagonists in blossoms. Concerns with using bees for primary establishment of bacterial antagonists include seasonal variation in the rate at which blossoms open and become colonized, dependence of

bee-foraging activity on weather conditions, presence of other flowering plants, which may draw inoculated bees away from the orchard, and the need for frequent monitoring of the pollen inserts to ensure that exiting bees are contaminated sufficiently with bacterial antagonists (Johnson *et al.*, 1993a). Because biological control of fire blight requires pre-emptive establishment of antagonist strains, variation in establishment of antagonists in blossoms when vectored by honeybees may be too high to provide consistent disease control. Moreover, data demonstrating control of fire blight as a result of bacterial antagonists being introduced into orchards by honeybees is currently lacking.

While honeybees may not be the best method to introduce antagonists into orchards, there is evidence that bees are an important mechanism in the secondary movement of bacterial antagonists from colonized to non-colonized pear blossoms (Nuclo *et al.*, 1998). As discussed above, this secondary movement of antagonistic bacteria can suppress epiphytic growth of *E. amylovora* and decrease the incidence of disease. The distinction between primary and secondary establishment of antagonists is important, and may be best viewed as analogous to the primary and secondary phases of a polycyclic disease epidemic, such as those caused by rust fungi in wheat. In this kind of process, primary colonization of supportive sites (e.g. leaves or stigmas) in a natural epidemic typically begins slowly and at low levels. As more and more sites become colonized, these sites in turn produce additional inoculum for secondary colonization. Most importantly, the rate of secondary colonization of new sites is maximal when colonized and non-colonized sites are present in roughly equal proportions (Zadoks and Schein, 1979). Spray applications, in contrast to honeybees, achieve primary establishment of bacterial antagonists quickly and in a relatively large proportion of blossoms. Thus, the high level of primary establishment of antagonists obtained with a sprayer reduces the time until secondary colonization of blossoms, which is facilitated by the bees, can occur at a maximum rate.

Stage of bloom at the time of application of antagonist strains is also an important consideration in achieving pre-emptive colonization of stigmatic surfaces (Nuclo *et al.*, 1998). Early bloom applications of antagonists are viewed as desirable because they provide the most time for the bacteria to establish and grow to large populations on stigmas before *E. amylovora* becomes active in the orchard. Complete protection of an orchard with an early bloom treatment, however, also requires that the bacterial antagonists spread from a small number of open blossoms treated directly with the suspension to a larger number of blossoms that open later in bloom. Conversely, applications made later in bloom are more likely to occur when the number of open blossoms is more favourable for antagonist establishment and subsequent secondary movement, but delaying application also risks prior or concurrent establishment by *E. amylovora*. Multiple applications of bacterial antagonists, at least twice between 25 and 90% bloom, appears to be the practical solution to this paradoxical problem. Temperature may also influence relative establishment of bacterial antagonists in blossoms, but the relationships are not completely understood. Nuclo *et al.*

(1998) observed poor establishment of *PfA506* and *EhC9-1* in 'Bartlett' pear blossoms applied at 15% bloom during a season when the mean daily temperatures averaged $< 6^{\circ}\text{C}$ for the following week. Similarly, cold weather (mean daily temperatures of $5\text{--}6^{\circ}\text{C}$) for 3 days following a single mid-bloom application of *PfA506* and *EhC9-1* on 'd'Anjou' and 'Bartlett' pear resulted in an incidence of recovery of the antagonists that averaged 20–25% in blossoms sampled after full bloom. In contrast, warmer weather (mean daily temperature of $11\text{--}12^{\circ}\text{C}$) following the same treatment on 'Bosc' and 'Comice' pear resulted in an incidence of recovery of *PfA506* and *EhC9-1* that averaged 80–85% after full bloom (K. Johnson, unpublished data). Again, multiple applications of antagonists between 25 and 90% bloom may be the most practical approach to enhance the probability that the antagonist suspensions are applied during periods when environmental conditions favour bacterial establishment.

Compatibility of bacterial antagonists with antibiotics

Successful integration of biological control into a conventional agricultural production system requires that the antagonist(s) used to suppress a plant pathogen will colonize the targeted surfaces of the crop consistently, and that these organisms will maintain effective populations during periods when other control methods (e.g. chemical applications) are employed. This aspect of the biological control of fire blight has been addressed in two research efforts (Lindow *et al.*, 1996; Stockwell *et al.*, 1996a), both of which concluded that the use of bacterial antagonists for fire blight suppression can be integrated with conventional (chemical) management strategies.

Lindow *et al.* (1996) reported on the efficacy of *PfA506* alone and in combination with antibiotic applications in pear orchard trials conducted over a 16-year period. These researchers found that populations of *PfA506* on pear blossoms were as high on trees treated with streptomycin as on trees treated with only *PfA506*, but that *PfA506* populations on blossoms treated with oxytetracycline were somewhat reduced. In small-scale field trials, the incidence of fire blight was reduced by 50% when treated with *PfA506*, by 40% when treated weekly with antibiotics (either streptomycin or oxytetracycline) and by 70% when a single treatment of *PfA506* was followed by weekly antibiotic treatments. Similarly, frost injury to pear blossoms was reduced by both *PfA506* and antibiotics, with the greatest reductions occurring when the chemical and biological treatments were combined. This research group concluded that biological and chemical control acted additively in the suppression of fire blight and frost injury.

Stockwell *et al.* (1996a), in studying population dynamics of bacterial antagonists in apple blossoms, also found that streptomycin, applied in a tank mix with bacterial antagonists or applied 2 or 7 days after the antagonist application, did not reduce either the incidence or mean population size of *PfA506* or *EhC9-1S* (a spontaneous streptomycin-resistant mutant of *EhC9-1*).

Furthermore, on some sampling dates, streptomycin in combination with *EhC9-1S* increased the population size of this bacterial strain in apple blossoms compared with the *EhC9-1S*-only control; this increase may have been caused by the suppression of competing, indigenous bacterial epiphytes that are sensitive to this chemical (Stockwell *et al.*, 1996a). Treatments of oxytetracycline, applied twice in mixture with streptomycin or applied as the first treatment in an oxytetracycline/streptomycin alternation, were detrimental to the population size and incidence of recovery of epiphytic populations of *PfA506* and *EhC9-1S*. Nonetheless, complete eradication of the antagonists following an oxytetracycline application was not observed in any of the experiments, which concurred with the observations of Lindow *et al.* (1996). Oxytetracycline suppressed populations of *PfA506* and *EhC9-1S* by slowing the rate of growth in colonized flowers and by reducing the rate of secondary movement to previously unopened blossoms, a result that is consistent with the bacteriostatic mode of action of this chemical. Oxytetracycline was most harmful to antagonist populations when applied 2 days after application of the bacteria, when many apple blossoms were still opening and becoming available for colonization. When oxytetracycline applications were delayed to 6–7 days after application of antagonist suspension, only slight reductions in the incidence of recovery or population size of *PfA506* and *EhC9-1S* were observed. Maintaining a delay of 6–7 days before initiating oxytetracycline treatments is relatively straightforward in orchards when streptomycin can be applied first. This delay strategy, however, may be more difficult to implement in production areas where the efficacy of streptomycin has been compromised by a high incidence of streptomycin resistance in the *E. amylovora* population.

Recommendations for use of bacterial antagonists in fire blight management

With the commercialization of *PfA506* and the potential registration of *EhC9-1S*, the following recommendation has been developed for the use of bacterial antagonists of *E. amylovora* in integrated fire blight management in the northwestern USA: *PfA506* (mixed with *EhC9-1S* if available) should be applied at least twice between 25 and 90% bloom for fire blight control. This recommendation is based on the knowledge that pre-emptive colonization of blossom surfaces by beneficial bacteria is essential to attain effective biological suppression of fire blight (Wilson and Lindow, 1993). Studies on the establishment and secondary movement of *PfA506* and *EhC9-1* also indicate the need for two applications of these bacteria for fire blight control (Nuclio *et al.*, 1998). In field trials conducted in the Pacific Northwest over several years, an application of *PfA506* and *EhC9-1* at 25–40% bloom, followed by a second application between 60 and 90% bloom, has suppressed fire blight in inoculated field trials by an average of 50–60% (Johnson *et al.*, 1993b; V.O. Stockwell, unpublished data). Similarly, in large-scale orchard trials conducted in California over several years,

two applications of *PfA506* at 20 and 90% bloom, in combination with a standard antibiotic control programme, reduced incidence of fire blight by 70%, when compared with the antibiotic programme alone (Lindow *et al.*, 1996). Two applications between 25 and 90% bloom will usually provide consistent establishment and thorough coverage of both early- and late-opening blossoms. Additional applications of bacterial antagonists after full bloom, particularly in combination with antibiotics, may add incrementally to control obtained with applications made before full bloom (Lindow *et al.*, 1996). Both *PfA506* and *EhC9-1* are resistant to streptomycin and can be tank-mixed with this chemical. Oxytetracycline sprays, however, should be delayed for 6–7 days after application of the bacteria antagonists (Stockwell *et al.*, 1996a).

Conclusions

The understanding and development of the biological control of fire blight has advanced to where this strategy is beginning to be used in commercial agriculture. Given current antagonist strains, biocontrol of fire blight, in most cases, should be viewed as a complementary disease control strategy, where the benefits from its use will be most significant when integrated with orchard sanitation and the application of antibiotics during periods of high infection risk. Two spray applications of bacterial antagonists of *E. amylovora* have typically provided 40–70% control, which is less control than that obtained when streptomycin is used to suppress streptomycin-sensitive strains of *E. amylovora*, but comparable to levels of control typically obtained with oxytetracycline. Successful use of biological control, however, requires pre-emptive establishment of antagonistic bacteria in blossoms, and thus growers may have to choose to utilize biological control before they know the risk of a fire blight epidemic during the current season. Some bacterial antagonists, notably *PfA506*, also reduce frost injury and russetting on the fruit surface, and thus these potential benefits should be weighed when considering the costs of using bacterial agents for fire blight control. Future research efforts are likely to identify new and more effective antagonist strains. In this regard, many of the considerations and methods outlined in this chapter will be applicable for the successful integration of these new antagonists into commercial orchard management.

There are several questions that remain to be answered regarding the development and use of biological control of fire blight for commercial agriculture. On the practical side, more information is needed on how environmental conditions in the orchard influence the establishment of spray-applied antagonistic bacteria in blossoms. Related to this is the question of whether environment-based warning thresholds for fire blight, which trigger antibiotic applications (Billing, Chapter 15; Steiner, Chapter 17), should be modified (i.e. raised) to incorporate the reduction in epidemic risk conferred by the use of biological controls. From a more basic point of view, we need to understand better how communities of antagonists interact on floral surfaces and whether these

communities can be manipulated or managed to enhance the efficacy of control. An improved understanding of the genetics and environmental fate of antibiotics produced by *E. herbicola* may also lead to strategies that will enhance the ability of these strains to suppress the epiphytic growth of *E. amylovora*.

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Integrated Orchard and Nursery Management for the Control of Fire Blight

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Introduction

Fire blight of apple and pear is incited by the bacterium *Erwinia amylovora* and has been known in North America for more than 200 years. Its control, however, has never been mastered to the degree that seems possible with other diseases of these and other valuable fruits and ornamentals in the *Roseaceae* family. Epidemics can develop rapidly in orchards with no history of the disease, destroying much of the current crop and killing many large limbs or whole trees within a few months. Epidemics also can be minor affairs, causing no significant economic damage, even in orchards that suffered severe blight the previous season. Between these extremes, variation in the incidence and severity of fire blight, which seems to follow no particular pattern from season to season and orchard to orchard, is characteristic.

Given the sporadic nature of fire blight, it is not surprising that some of our management tactics sometimes fail to provide consistent control. There are instances, for example, where a significant amount of blossom blight occurs despite a grower's best efforts to follow a recommended programme of orchard sanitation and a series of protective antibiotic sprays during bloom. There are also many seasons when a similar spray programme seems excessive, given the small amount of disease that occurs, in nearby untreated orchards. Even when no blossom blight occurs, we sometimes see damaging epidemics of shoot blight and many cases where hailstorms trigger severe outbreaks. In fact, shoot infections, for which there is currently no effective alternative to eradication for control, pose the most common risk in fruit-tree nurseries.

Constraints to effective fire blight management

Managing fire blight well is difficult because our tactical options are limited largely to cutting out infected limbs and applying copper-containing formulations or antibiotics. Unfortunately, copper materials are often phytotoxic, antibiotics are really only effective against blossom infections and cutting can be inefficient when the amount of disease is high. The use of antibiotics is also limited in some areas because resistant strains of the pathogen are present (Stockwell *et al.*, 1996; see also Jones and Schnabel, Chapter 12) or because government regulations in some countries prohibit this practice.

Changes in modern orchard management practice and market demand over the last two decades have pre-empted the widespread use of resistance and reduced fertility as options for lessening the risks of fire blight. At the same time, they have increased the vulnerability of many orchards. In apples, for example, instead of planting 250–500 trees ha⁻¹, growers now set trees at up to ten times that number. Such high densities require the use of size-controlling rootstocks, of which the two most widely used, M.26 and M.9, are highly susceptible to fire blight. Adding to the risk of loss in many areas is an increase in hectares planted to new fresh-market apple varieties, such as ‘Gala’, ‘Fuji’, ‘Braeburn’ and ‘Granny Smith’, along with older favourites, such as ‘Rome’, ‘Ida Red’ and ‘Jonathan’, all of which are very susceptible. Finally, to maximize production efficiency in these high-density orchards, strong vegetative tree growth is encouraged in young plantings so that trees fill their allotted space within 3 years. Various methods of tree training are then used to induce flowering at the expense of vegetative growth, so that infections often lead to more limb and tree death than generally experienced with larger trees (Suleman and Steiner, 1994).

Integrated disease management

Integrated disease management is the knowledgeable selection and use of all appropriate strategies and tactics required to suppress the damage caused by pathogens to some economically acceptable threshold. The ‘acceptable threshold’ for fire blight, however, must be very low, since even a very low incidence of primary infections can fuel the development of an explosive epidemic, with the potential to destroy whole orchards. In this chapter, I discuss the design of an aggressive fire blight management programme that uses conventional tactics, but with improved timing and some slight modifications. In principle, this approach focuses on reducing the number and distribution of inoculum sources on a continuing basis throughout the season, every year, regardless of the amount of disease present. The programme relies on the MaryblytTM forecasting program (Steiner, 1990a, b; Steiner and Lightner, 1996) as an aid in timing protective sprays and orchard sanitation practices for maximum impact. Van der Zwet *et al.* (1988) reviewed the use of several other model systems for

predicting fire blight risks, but all dealt only with blossom infections and none identified specific infection events or predicted symptom appearance. MaryblytTM is unique in its identification of specific infection events for four different types of infections, as well as the appearance of symptoms associated with those events. The more recent Billing's integrated system (Billing, 1996) and another model proposed by Smith (1995) incorporate some aspects of the MaryblytTM model, but still deal only with the blossom blight phase of epidemics. A review of all these programmes is presented by Billing (Chapter 15).

Significance of multiple infection types

When an epidemic of fire blight unfolds, there is a tendency to see only the collective damage, as the number of infected limbs and trees increases. In developing the MaryblytTM forecasting model, Steiner (1990a, b, 1991) defined five distinct types of infections caused by *E. amylovora* that contribute to the wide range of symptoms that occur in fire blight epidemics. These types give rise to symptoms called blossom, canker, shoot, trauma and rootstock blight (Table 17.1). Not all infection types occur in every orchard every year or even with the same intensity, but all of these, except rootstock blight, can be predicted. These types differ in the sources of inoculum, the conditions required for infection, the tissues attacked and the appearance of early symptoms. Once epidemics are well under way, the characteristic symptoms of individual infection types and separate events become harder to distinguish, as many infections continue to progress, invading larger portions of the tree. Understanding these differences in how and when these infections occur is important in the selection and use of the most appropriate control tactics at the right time.

Blossom blight

Early random dispersal of primary inoculum from overwintering cankers by wind, rain splash and casual insect activity up to several weeks prior to bloom, sets the stage for sometimes explosive epidemics (Miller and Schroth, 1972). Once the first flowers open, dispersal of the pathogen is no longer random but is directed specifically to open flowers by pollinating insects. Here the flower stigmas are selectively colonized by *E. amylovora* to very high levels (Thomson, 1986) and the proportion of open flowers increases exponentially as a function of time and temperature (e.g. degree-hours (DH) > 18.3°C (Zoller and Sisevich, 1979)). The four minimum requirements defining an infection event in the current version of MaryblytTM (Steiner and Lightner, 1996) are: (i) flower buds open (to allow colonization of stigmas) and with petals intact (flowers in petal fall are resistant); (ii) accumulation of at least 110 degree-hours > 18.3°C within the last 44 or 66 degree-days (DD) > 4.4°C for apples and pears, respectively; (iii) a

Table 17.1. Characterization of the multiple types of symptoms and infections associated with fire blight epidemics.

Type	Primary infection court	Conditions for infection	Characteristic early symptoms
Blossom blight	Nectarhodes of open flowers with intact petals	1. Open flowers with intact petals 2. Accumulation of 110 degree-hours (DH) > 18.3°C during last 44 (apple) or 66 (pear) degree-days (DD) > 4.4°C 3. Wetting event as rain or dew ≥ 0.25 mm or ≥ 2.5 mm the previous day 4. Average daily temperature ≥ 15.6°C	Bacterial ooze droplets or dark streaks on flower petioles; wilt followed by necrosis of flower cluster
Canker blight	Cortical and xylem parenchyma surrounding overwintering cankers	Presence of overwintering cankers with indeterminate margins; accumulation of approximately 52 degree-days > 12.7°C after green tip stage	Narrow (≥ 1 mm) water-soaked zone in green bark tissue adjacent to necrotic tissue at canker margin regularly coincident with accumulation of 109 degree-days > 12.7°C after green tip; at approximately 166 cumulative DD (CDD) >12.7°C after green tip, vegetative shoots near cankers show yellow-orange discoloration of tip bud before wilting and basal leaves may show dark streaks in petiole and mid-vein
Shoot blight	Top one to two leaves of vegetative shoots	Usually presence of either blossom or canker blight symptoms; wounding event caused by insect feeding (?) or gusty winds (?); average temperature ≥ 15.6°C	First shoot tip infections appear as wilt of vegetative shoot tip, which initially remains green, but usually third leaf from tip shows necrosis along basal mid-vein and into leaf lamina; most often coincident with accumulation of 57 DD >12.7°C after appearance of blossom or canker blight symptoms; subsequent shoot tip infections appear more or less at random

Trauma blight	Non-specific; includes leaves, fruit, bark	Accumulation of 110 DH >18.3°C over last 44 DD > 4.4°C; wounding event caused by late frost ($\leq -7^{\circ}\text{C}$), hail or high winds that damage foliage (wetting is not required, but may increase number and severity of infections)	Necrosis of foliage apparent 57 CDD > 12.7°C after trauma event; if damage occurs at or before petal fall, symptoms may appear as blossom blight, but spur leaves are more necrotic when wilt occurs than with normal blossom infections
Rootstock blight	Xylem parenchyma cells in rootstock bark (especially M.26 and M.9 but can affect M.7 and M.111, others?)	Infection of blossom clusters or shoots on scion variety	(i) Bacterial ooze evident on bark surface of rootstock; (ii) rapidly enlarging cankers on rootstock; (iii) sudden collapse of scion in mid-season; (iv) early red autumn colour on scion leaves in late season; (v) canker development upward into scion trunk with subsequent death of tree in spring following infection

DH, degree-hours; DD, degree-days

wetting event ≥ 0.25 mm as rain or dew or ≥ 2.5 mm the previous day; and (iv) an average daily temperature of 15.6°C .

When all four of the above conditions are met in the sequence shown, infections probably occur within minutes and early symptoms appear regularly with the accumulation of an additional 57 DD $> 12.7^{\circ}\text{C}$ which, in real time, can range from 5 to 30+ calendar days. The MaryblytTM program will track up to ten separate blossom infection events simultaneously and the symptoms from infection events as little as 1–2 days apart can be distinguished. A single blossom infection event can introduce thousands to hundreds of thousands of new sources of inoculum, which are then available to fuel a continuing epidemic of secondary flower and vegetative shoot infections. Secondary flowering in pears and some apple cultivars can prolong the high risk for blossom infections in many areas (Deckers and Daemen, 1993).

Canker blight

Canker blight refers to the renewed infectious activity of *E. amylovora* overwintering in the live bark tissues just beyond the margins of those cankers established the previous season which are not isolated by a barrier of suberized cork cells (i.e. indeterminant margins, *sensu* Miller (1929)). Estimates based on the fixed infection-to-symptom interval of 57 DD $> 12.7^{\circ}\text{C}$, which is consistent among all predictable types of infections, suggest that this renewed activity at canker margins begins approximately 52 DD $> 12.7^{\circ}\text{C}$ after the green tip stage of bud development, which is generally coincident with the tight cluster stage to early pink bud stage in apple or white bud stage in pears (Steiner and Lightner, 1996). Suleman and Steiner (1994) speculated that this activity may be triggered by the mobilization of soluble reserve carbohydrates (mostly sorbitol) in response to early vegetative growth.

In those seasons when blossom blight occurs, the relatively minor loss of a few shoots or limbs as a result of canker blight is probably insignificant as a continuing source of inoculum. In years when blossom infections do not occur or are well controlled, however, the appearance of canker blight symptoms may be a key source of inoculum that contributes to a continuing risk for shoot and trauma blight (Steiner, 1990b). In general, where blossom blight is not a factor and the number of active cankers is small, the distribution of secondary shoot blight symptoms is usually localized at first near cankers, but redispersal from those new sources through wind, rain and insect activity can increase the risks for subsequent shoot and trauma blight over much larger areas.

Shoot blight

Shoot blight refers to infections that are initiated on the tip leaf or leaves of vegetative shoots, which then progress downward, often invading and killing a

portion of the supporting limb. The appearance of even a few shoot blight symptoms represents a new source of epiphytic inoculum, which can be dispersed by wind and rain to other leaves in the orchard and especially to areas on the same tree directly below earlier infections. While there has been much speculation on the role of sucking insects in initiating shoot tip infections, there is little documentation supporting such a key role. A more likely scenario may be that epiphytic populations of the pathogen vary with prevailing weather conditions over the course of the season and that injuries to the tip leaves caused by insect feeding or whipping by gusty winds may open tiny wounds that allow surface bacteria easy entrance (Bauske, 1967; Bogs *et al.*, 1998). Indeed, Bogs *et al.* (1998), using bioluminescent and fluorescent genetic markers, documented the entry of *E. amylovora* through tiny wounds at the base of trichomes on immature pear leaves; it then moves rapidly to other parts of the plant. In this sense, then, shoot blight may be little more than trauma blight on a much smaller scale.

Trauma blight

Trauma blight is a term coined by Steiner (1990b) for the non-specific infections of leaf, fruit and bark tissues associated with injuries caused by late frosts $\leq -2^{\circ}\text{C}$, hail or high winds. Its widespread occurrence throughout an orchard, especially when preceded by the appearance of blossom, canker or shoot blight symptoms, strongly supports the concept of epiphytic populations of the pathogen that persist through much of the season. Since wounding may breach normal plant defence mechanisms (Suleman and Steiner, 1994), trauma blight can result in severe damage even on highly resistant cultivars, such as 'Red Delicious'. Eldon Zehr (Clemson University, 1988, personal communication; see also Steiner, 1990b) observed nearly 80% of the shoots on 'Red Delicious' and 'Golden Delicious' apples blighted following a late frost.

Rootstock blight

Rootstock blight in apples involves damaging secondary infections that occur when bacteria from strikes in the scion variety move through an otherwise healthy limb and trunk structure into the rootstock, where new bark cankers are initiated and subsequently girdle and kill all or part of the tree (Steiner, 1991). It occurs most frequently with the M.26 and M.9 rootstocks and C.6 interstems when fire blight susceptible scion cultivars become infected (blossom, shoot or trauma blight), but it can also occur with resistant scion cultivars following a trauma incident. Rootstock blight also occurs on M.7A and M.111 apple rootstocks (P.W. Steiner, unpublished observations), but the cankers do not appear as aggressive as on the more susceptible rootstocks and they rarely kill trees. Suleman (1992) used a strain of *E. amylovora* doubly resistant to

streptomycin and rifampicin antibiotics and noted the appearance of the bacteria in M.26 rootstocks of 1-year-old 'Red Delicious' and 'York' apple trees within 10 days after the inoculation of a single vegetative shoot on the scion variety. Bogs *et al.* (1998) and Momol *et al.* (1998) recently documented this phenomenon in more detail. Bogs *et al.* (1998), in fact, offer proof that *E. amylovora* moves downward rapidly in xylem elements and can initiate new infections in the xylem parenchyma tissue of the rootstock just below the graft union. Recent field observations by this author (1998, unpublished) on first year 'Gala' apple on M.26 rootstocks indicate that most rootstock cankers appear to be initiated 10–15 cm below ground level regardless of how high the graft union may be above ground.

In the relatively warm mid-Atlantic area of the USA, symptoms of rootstock blight can appear in three phases. Trees may die suddenly in mid-season, another group of trees show symptoms of early red autumn colour in late August to early September and a final group of trees show symptoms of poor development in the early spring of the year following infection. These latter trees usually exhibit distinct cankers, which progress up from the rootstock into the trunk. In the cooler climate of New York State, only the latter two symptom types of rootstock blight are common (Momol *et al.*, 1998). Unfortunately, we still lack key information on the physiological and environmental factors determining if and when rootstocks cankers develop on specific trees, because not all trees showing scion infections later succumb to rootstock blight. Although Momol *et al.* (1998) indicate that a higher proportion of trees infected late in the season develop rootstock blight, there have been instances where infections at or just after petal fall also lead to high losses due to rootstock blight (P.W. Steiner, 1990, unpublished observations). In the Appalachian area in the USA (Pennsylvania, Maryland, Virginia and West Virginia), we generally see an average of 5–6% of the trees on susceptible rootstocks that show symptoms of scion infection (blossom, shoot and trauma blight) die each year over the first 5 years after planting. Losses as high as 60–80% of the trees, however, have been observed in some orchards over a 2-year period, especially following a trauma blight situation incited by hail or high winds. Similar losses occur in New York State (Momol *et al.*, 1998).

Aggressive fire blight management programme for orchards

Nearly all recommendations for fire blight management begin with the thorough removal of all infected limbs during the dormant pruning operation. This is followed by an early-season, full-coverage spray with either Bordeaux mixture or any one of several fixed copper formulations to reduce the efficacy of any remaining inoculum. Major emphasis is also given to the use of a series of sprays to prevent blossom infections. These are applied frequently during bloom using fixed copper materials, antibiotics, such as streptomycin or oxytetracycline, or other chemicals, such as flumequin (FirestopTM, FructilTM), in Europe (Deckers

et al., 1990). After bloom, about the only effective tactic for limiting the damage caused by fire blight is to cut out new infections when symptoms appear (Covey and Fischer, 1990). The aggressive fire blight management programme presented here has been tried and amended over the last decade in conjunction with the development of the MaryblytTM blight computer forecasting program. Our experience in monitoring the implementation of this program by growers is that usually, within 3 years, overall control of fire blight is fairly consistent and the risks for major tree and limb losses are so reduced that, even following severe hail damage, most orchards sustain only minor amounts of fire blight.

Dormant pruning

Dormant pruning is routinely done during the winter to maintain a high proportion of fruiting wood and to control tree size and shape. Removing all visibly infected limbs and, where necessary, whole trees is absolutely imperative for any fire blight management programme to reduce the number and distribution of primary sources of inoculum. In practice, many small cankers are overlooked, while others on large limbs may be left deliberately on the chance that they will not become active. Close attention is also given to the removal of all 'ugly stubs', which have a high potential for harbouring small, almost cryptic cankers induced to form around cuts made to remove infected branches the previous season. Details of this latter procedure are discussed later. In years following late-developing epidemics, a higher proportion of infections result in the formation of cankers of the type most likely to be active the following spring (Beer and Norelli, 1977; Biggs, 1994).

Early-season copper spray

An early-season copper spray is recommended for application at the green tip stage of apple and pear bud development. Many conventional recommendations refer to a 'dormant' copper treatment. In the course of constructing the MaryblytTM model, a regression analysis of observations on the timing of symptoms developing at canker margins and, later, on nearby shoots suggested that infectious activity around the margins of overwintering cankers probably occurs regularly with the accumulation of about 52 DD > 12.7°C after green tip. This timing is usually coincident with the tight cluster to early pink phenological stage on apples (Steiner and Lightner, 1996). Thus, the rapid multiplication of the pathogen around overwintering canker margins is most likely to contribute to the elaboration of bacterial ooze on to the bark surfaces 1 to 2 weeks after bud break. This means that the active copper residues from sprays applied when the trees are still dormant may undergo as much as a month of weathering before bacteria are readily available on the treated surfaces. By delaying the application until just after bud break, but before the 1.25 cm green

stage, when phytotoxicity may be a problem, our aim is to have an abundance of copper ions available as an effective barrier to reduce the efficacy of the bacteria in colonizing bark and bud tissues during the pre-bloom period.

Equally important is the fact that, since inoculum dispersal in an orchard by wind, rain splash and insect activity prior to blossoming is largely a random event, it is entirely likely that some epiphytic populations of the pathogen are established on any tree in the orchard, irrespective of its inherent susceptibility to fire blight. Thus, when copper is applied at the beginning of the season, the application should include all host trees in the orchard, not just those known to be susceptible to fire blight. Failure to do this may allow epiphytic populations of the pathogen to build up on resistant varieties, such as 'Red Delicious', and then be moved to susceptible blossoms by insects, completely bypassing the copper residue. When the copper spray is delayed until the green tip stage, it is effective as a replacement for fungicides used at that time for apple scab control. Furthermore, by combining the copper with a 2.0% superior oil for early season mite and aphid control, coverage with the copper is enhanced, while improving the overall efficiency of the orchard pest control programme.

Protective blossom sprays

Most conventional spray programmes for preventing blossom blight use a series of treatments made at regular 4- to 7-day intervals during the bloom period. This approach, while generally adequate, sometimes fails when risks are high and often seems excessive when risks are low. Even a 24 h delay in treatment with streptomycin after an infection event, for example, can result in only 90% control which, near full bloom, can result in a substantial number of infected blossom clusters (W.H. Shaffer, Jr, 1989, University of Missouri, personal communication). The specific targets for treatment are the flower stigmas, which can be thoroughly colonized by *E. amylovora* in as few as 2–3 days after opening, and the nectarthodes, which are the primary sites of infection (Thomson, 1986). Neither of these critical targets is exposed until flower buds open and, of course, spray coverage must be adequate to ensure that all such targets are protected. Treated flowers are protected for the life of the open blossom, which is approximately 44 and 66 DD > 4.4°C for apples and pears, respectively (Steiner and Lightner, 1996). Once these flowers begin to senesce and drop petals, however, the nectarthodes are no longer functional and the flowers appear naturally resistant. Precise timing of spray treatments is possible with the MaryblytTM forecasting program, so that sprays can be applied only when needed and then just prior to an anticipated infection event. When this is done, nearly complete control is possible, often with fewer treatments than with most conventional approaches. In Maryland, where we have used MaryblytTM regularly for 10 years to make recommendations for fruit growers, only one or two sprays per year are usually needed, sometimes no treatments are recommended and rarely is there a need for three or more sprays. In other areas, where secondary or 'rat-

tail' bloom occurs regularly, more treatments may be necessary but, even then, they can be made only when needed.

The second critical factor in the efficient control of blossom blight is thorough spray coverage. High-volume sprays of dilute (e.g. 100 p.p.m. streptomycin) spray mixtures that approach the drip point generally require 1870–2800 l ha⁻¹, but they also require more time and labour than low-volume sprays. Low-volume sprays, using 370–560 l ha⁻¹, apply a more concentrated mixture (e.g. 300–400 p.p.m.), which is generally adequate for coverage and control in most orchards. In Maryland, I have discouraged the use of pesticide dosages based on kg ha⁻¹ rates in favour of specific concentration and spray volume factors, which are more relevant to a three-dimensional orchard target (Steiner, 1986). With this system, the concentration and spray volume rates recommended for fire blight in Maryland for high-volume and low-volume sprays, respectively, are 100 p.p.m. applied at 9.3 l 100 m⁻³ of tree row volume ha⁻¹ and 300 p.p.m. applied at 1.2 l 100 m⁻³ of tree row volume ha⁻¹. Here, tree row volume is defined as tree height (m) × tree width (m) × tree row length ha⁻¹ (m) (= m³ tree row volume ha⁻¹). In most cases where an activator spray adjuvant is used, the above concentrations can be reduced by half.

While our experience with the above rates and timing of applications has been largely with the antibiotic streptomycin, the principles involved should apply equally well to most other materials where streptomycin cannot be used due to either resistance problems or regulation. In Maryland, for example, where all pesticide dosage rates have been routinely recommended in low-volume orchard sprays since 1984, these guidelines on spray volume and concentration have proved effective with all insecticides and fungicides used in orchard pest control. The only exception with respect to fire blight control may be with the newly registered material, fosetyl-Al (AlietteTM). Here, fosetyl-Al is presumed to trigger plant defence mechanisms, so that treatments need to be scheduled on a phenological basis every year, rather than in response to identified risk periods, as is done with most other materials. This would also apply to other materials which induce systemic acquired resistance such as ActigardTM (= BionTM in Europe, BASF) or MessengerTM (= harpin, Eden Bioscience) (Momol *et al.*, 1999).

Perhaps the most dramatic effects on reducing the incidence of secondary shoot blight infections have come with the use of a new synthetic gibberellin biosynthesis inhibitor, prohexadiore calcium (ApogeeTM, BASF) which is soon to be registered for use in the USA in early 2000. Applied between full bloom and petal fall on apple, ApogeeTM has consistently reduced internodal length of vegetative shoots; a reduced incidence of shoot blight under field conditions, and a reduced rate of tissue invasion follow shoot inoculation. This activity appears heightened with the addition of ammonium sulphate to the spray mixture (Yoder *et al.*, 1999).

Biological control

Two bacterial antagonists have shown good activity in protecting against blossom infections (see Johnson and Stockwell, Chapter 16). Strain A506 of *Pseudomonas fluorescens*, marketed since 1995 as BlightBan A506™, multiplies rapidly, so that it effectively excludes subsequent colonization by *E. amylovora*, so long as it reaches the flower before the pathogen (Wilson and Lindow, 1993). In early 2000, however, the BlightBan A506™ product was temporarily withdrawn from the market by the manufacturer. Subsequent dispersal of the antagonist to other opening flowers occurs through the activity of honeybees (Johnson *et al.*, 1993). Another biological antagonist, *Erwinia herbicola* C9–1, produces at least two antibiotics that limit the growth of *E. amylovora* and, like *P. fluorescens* and *E. amylovora*, is also dispersed by honeybees. Tests with this second organism show similar levels of control of fire blight, but it has not yet been registered for use by growers.

Lindow *et al.* (1996) showed that the effects of *P. fluorescens* A506 and antibiotics were additive with respect to fire blight control in pears. In the western USA, where a majority of *E. amylovora* isolates are resistant to streptomycin, combinations of *P. fluorescens* and *E. herbicola* C9–1 may provide a welcome alternative to streptomycin. A second antibiotic, oxytetracycline, has recently been registered for use in the western USA and other areas where streptomycin resistance is a problem. Stockwell *et al.* (1996) note that populations of both antagonists are sensitive to oxytetracycline and suggest that its use be delayed until at least 7 days after the antagonists are introduced to reduce this effect. In the eastern USA, where streptomycin resistance in *E. amylovora* is not a major problem, Hickey and van der Zwet (1995) found that *E. herbicola* (strain 318) provided 65% control of blossom blight and was equal in performance to streptomycin alone in tests where trees were challenge-inoculated with the pathogen. In the same tests, however, *P. fluorescens* A506 did not significantly reduce the incidence of fire blight. Precisely how these new antagonists and others under study will ultimately be used will require more regionally adapted research before broad recommendations can be made. Nevertheless, the best overall approach is likely to include the use of a combination of two or more antagonistic organisms at early and full bloom, coupled with the selective use of compatible antibiotic treatments just prior to predicted blossom infection events. It should be noted that *P. fluorescens* A506 and *E. herbicola* C9-1 are only the first widely tested biological antagonists for fire blight control. Pusey (1997) lists a number of other antagonists under study, some of which appear to have high levels of activity, but have yet to be released for commercial development.

Cutting out active infections

Conventional recommendations for removing infected shoots and limbs during the course of the season generally instruct the grower to make cuts at a healthy

branch union at least 20–30 cm below any visible symptoms, using pruning tools surface-sterilized between each cut with copper, alcohol or bleach solutions. When this is done, Clarke *et al.* (1991) found that 12% of the cut stubs left in the tree harboured *E. amylovora*. In practice, most cuts are made back to a healthy branch union, as is typically done when pruning during the dormant season. Observations by Suleman (1992) indicate that the bacteria can be isolated up to several metres ahead of any visible symptoms during the growing season, even though the tissues at such remote locations are symptomless. P.W. Steiner (unpublished data, 1990) observed new canker development around many cutting wounds on pears in the US Pacific Northwest 2–3 weeks after infected branches were cut out, even where growers routinely treated all pruning tools with a copper sulphate mixture between cuts. P.W. Steiner (unpublished data, 1990, 1991) later found that small, nearly cryptic, cankers often less than 0.25 cm, frequently developed around the wounds made in removing infected shoots, even where both the pruning tools and the bark where the cut was made were surface-sterilized. In the spring following such cuts, infectious activity was initiated, which produced early symptoms of canker margin extension coincident with the accumulation of 109 DD > 12.7°C after green tip.

Based on these results, and the fact that surface sterilization of tools made no difference in preventing small canker formation, a modified cutting procedure, called ‘ugly stub cutting’ was developed. In this procedure, growers are still advised to make cuts 20–30 cm below visible symptoms in wood that is at least 2 years old (resistance is attributed to greater carbohydrate reserves in the bark of older branches (Suleman and Steiner, 1994)), using unsterilized tools. However, instead of making the cut as usual at a healthy branch union, the cut is made leaving a 10–12 cm naked stub. While small cankers will form on many of these cuts, they can be removed during the winter pruning operation. In practice, many growers routinely mark all newly cut ‘ugly stubs’ with bright orange spray paint, so they can be located more easily in the following winter. This is an important modification for two reasons. First, by not sterilizing all cutting tools between cuts, the time required for the cutting operation is reduced by more than half, which means not only lower costs, but also that the job can often be completed quickly using fewer workers. Secondly, the cankers that develop at the tip of the ugly stub can be easily and completely removed from the orchard before they can supply new primary inoculum for the next season. Travis and Kleiner (1997) demonstrated little effect in the number of active cankers formed on cut stubs when tools were or were not sterilized and when cut surfaces were or were not chemically treated.

Prompt removal of infected shoots as soon as symptoms can be detected is just as important as how the cuts are made. The longer infected shoots remain in the orchard, especially if rain follows symptom appearance, the greater the chances for secondary inoculum dispersal to other locations in the trees and throughout the orchard. Indeed, Covey and Fischer (1990) reported that delaying the initial cutting by 2 weeks after first symptoms appear resulted in the removal of six times more plant material over the course of the season. The

Maryblyt™ program can be indispensable in the early detection of symptoms, since it is quite accurate in this regard (Jones, 1992; McManus and Jones, 1994; van der Zwet *et al.*, 1994). This greatly improves the efficiency of orchard monitoring efforts and encourages the timely removal of infected spurs, shoots and limbs.

If the symptoms from early infection events can be removed quickly, we often see relatively few secondary shoot infections in well-managed orchards. This can be very important, since cankers that form later in the season are more likely to be active sources of inoculum the following season (Beer and Norelli, 1977; Biggs, 1994). Indeed, Biggs (1994) indicates that the chances for the formation of indeterminate cankers following the cutting procedure are > 80% on apples after mid-June in West Virginia. All cut branches should be removed from the orchard and destroyed, since they may continue to provide bacterial ooze on their surfaces, which can then be redispersed by insects and wind-driven rain through the orchards.

Summary on orchard management

The aggressive fire blight management approach described here focuses on strict orchard sanitation in both the dormant and growing seasons, coupled with the use of well-timed protective sprays during the flowering period. The principal differences between this and other, more conventional, approaches lie in the precise timing of each spray and cutting operation for maximum effect, the manner in which new infections are removed and the close attention to orchard sanitation every year, regardless of how few infections might occur. Growers following such practices generally acknowledge that, within 2–3 years, they feel ‘comfortable’ that their risks for catastrophic losses, even under severe conditions, such as hailstorms, are considerably reduced. Indeed, the outstanding success of blossom blight control programmes using a good forecasting programme to time spray applications has almost eliminated any serious blossom blight problems. This, in turn, has apparently reduced the incidence of secondary shoot tip infections so that those infections, that do occur can generally be quickly removed with a minimum amount of labour within 1 day or less over a 30 ha orchard.

Fire blight management for nurseries

Fire blight poses significant problems both for nurserymen and for growers who purchase trees from nurseries where blight occurs. Since the pathogen can remain latent in symptomless tissues (McManus and Jones, 1994; Crepel *et al.*, 1995), its unintentional introduction into nursery fields through contaminated bud wood and into growers’ orchards in newly purchased, dormant trees can be especially troublesome. McManus and Jones (1994) emphasized the

importance of keeping nursery stock free of *E. amylovora*, because of the potential of not only introducing the pathogen into areas where blight is not known, but also introducing new genotypes of the pathogen in areas where the disease is already established. This latter issue warrants serious concern, since strains of the pathogen resistant to the antibiotic streptomycin can pre-empt the use of this material in controlling the disease in areas where it might otherwise be effective.

While blossom blight is particularly damaging in commercial orchards, it is not generally a problem in nursery fields, but the abundance of succulent vegetative growth and the wounding that occurs as part of routine nursery practices, such as bud and shoot removal, provide ample opportunities for shoot and trauma blight incidents. In addition, since the pathogen is readily transported as aerosols with even modest wind-driven rains at $3\text{--}6\text{ m s}^{-1}$ (McManus and Jones, 1994), the presence of even a few sources of inoculum can be enough to initiate major epidemics of secondary shoot blight in large nursery fields planted at high densities of more than $20,000\text{ trees ha}^{-1}$. Thus, the challenge here is to design guidelines for limiting the introduction of *E. amylovora* into and its spread within nursery fields in ways that are practical, easy to implement and economical. To my knowledge, there are no published accounts that evaluate the efficacy of holistic fire blight control programmes in apple and pear nurseries. The following guidelines, therefore, are based largely upon basic principles of plant disease management, work by Bauske (1967) and McManus and Jones (1994) and the author's experience with several tree-fruit nursery operations.

Guidelines for excluding *E. amylovora* from nursery fields

1. Locate nursery production fields away from producing orchards and, ideally, in areas where apples and pears are not grown commercially.
2. Maintain bud-wood source orchards separate from tree production fields, isolated from producing orchards, and manage them in ways that produce an abundance of vegetative growth and little or no flowering. Where spurious flowering does occur in these source orchards, remove flower-bud clusters by hand every spring before bloom occurs.
3. Where bud wood must be obtained from producing orchards, limit bud-wood selections to sites where fire blight either does not occur or is generally well managed. In some high-risk situations, it may be prudent to manually deflower and enclose selected trees for bud-wood harvest in hail netting, in order to reduce the potential for wind and storm damage in the summer prior to bud-wood harvest.
4. Since the greatest risk for introducing *E. amylovora* may be through contaminated bud wood, separate plant production fields should be maintained for trees grafted using only buds from the home nursery source orchard and for those using buds from growers' orchards, which pose a higher risk for contamination. Obviously, as workers move between these two fields, all hand tools should be

routinely disinfested in an alcohol or bleach solution. For the same reason, where workers are routinely removing buds and shoots by hand in these orchards, they should wash their hands thoroughly when moving to another field.

Guidelines for monitoring and eradicating infected trees

1. All production fields should be monitored regularly for the appearance of fire blight symptoms throughout the season, but especially after any symptoms appear in the general area outside the nursery and always after any hail or wind-driven rainstorms. Here, the simulator in the MaryblytTM program can be useful in scheduling timely monitoring efforts for periods when early symptoms of infection are most likely to be found. McManus and Jones (1994), for example, observed fire blight symptoms regularly on or within 3 days after the date predicted using the MaryblytTM computer program.
2. Any tree showing any infection should be uprooted promptly and placed in a large plastic bag before carrying it out of the field. Prompt and complete removal of infected trees is the key here, since the longer the delay, the greater the chances for secondary dispersal of the pathogen to other trees in the vicinity. Trees that are simply cut off at the soil line, often produce new shoots, which can be systemically invaded by the bacteria and continue to provide inoculum within the planting. Because infected trees generally have high populations of the bacteria on leaf and stem surfaces, carrying uprooted trees to the edge of a field without first bagging them may contaminate other trees in the row.
3. Use flags to mark all areas of the field where blighted trees are removed and schedule these areas for additional close inspection to detect new infections not previously visible.

Guidelines on preventing infection

1. Bauske (1967) noted that the severity of fire blight was in direct proportion to the degree of exposure of pear trees in nursery fields to the prevailing southerly winds in Iowa and that there was a distinct gradient in the amount of disease recorded with distance from a Lombardy poplar windbreak. He also noted the increased susceptibility of young shoot tip leaves to infection following exposure to simulated winds of 6 m s^{-1} . More recently, McManus and Jones (1994) documented the incidence of apple shoot tip infections in nursery rows, which was correlated with the occurrence of winds as low $3\text{--}6 \text{ m s}^{-1}$, but especially following wind-driven rainstorms with maximum 1-min mean wind speeds of $8\text{--}9 \text{ m s}^{-1}$. On this basis alone, it seems that, wherever possible, nurserymen should consider the use of windbreaks orientated perpendicularly to the prevailing winds along nursery fields to reduce the incidence of shoot tip infections.
2. Once any fire blight is detected in a field, it seems only prudent to plan on

the application of a suitable fixed copper formulation 1–2 days prior to any scheduled operation where workers will be rubbing or pinching off buds, leaves or small shoots by hand. The purpose of these treatments is not as a preventive for shoot blight, but to reduce the risks of infections associated with the wounding process of shoot and bud removal. This recommendation is based on the findings of Crosse *et al.* (1972), who noted that leaf damage exposing xylem tissue predisposed tissues to infection on apple shoots, and the recent findings of Bogs *et al.* (1998) regarding entry of the pathogen through damaged leaf hairs. In addition, the author observed that seven of 15 infected trees removed from one apple nursery field in 1997 showed infection foci at the point where succulent sucker shoots had been pinched off by workers. Use of copper on a regular or prophylactic basis should be avoided, since cumulative residues can lead to partial defoliation, which, in turn, can reduce the stem caliper or grade standard for nursery trees. Antibiotics, such as streptomycin, have not proved effective in reducing shoot blight when used in protective sprays (Suleman, 1992) and should not be used in place of copper, because of the added risk of selecting resistant strains of the pathogen. With additional testing, materials such as flumequin (FirestopTM) or a systemic acquired resistance inducing material (ActigardTM, BionTM, AlietteTM) may have a role here, although such systemic acquired resistance (SAR) compounds will need to be applied at least a week or more before a scheduled bud or shoot removal operation.

While the MarybltTM computer program can be useful in detecting the earliest shoot blight symptoms, it is not effective in predicting the appearance of subsequent secondary shoot infections. These latter events appear to occur at random through the growing season and may be triggered by wind abrasion (wind-blown sand or leaf-to-leaf abrasion of trichomes) at moderate speeds and by various insects feeding on newly emerging, immature leaves. Given this lack of detail on when specific shoot tip infection events occur and the distinct possibility that they may occur at a high frequency in some years, the economic prospects of preventive chemical treatments here seems remote. However, new developments in the area of specific acquired resistance elicitors such as harpin (Wei and Beer, 1995) and ActigardTM (Novartis Corp., Greensboro, North Carolina, USA) may provide an effective management tactic here in the future.

Summary on fire blight management in nurseries

The success of any fire blight management approach in nurseries will hinge on the implementation of rigid guidelines designed to exclude *E. amylovora* from nursery fields and, where it does occur, additional guidelines for the early detection and complete eradication of infected plants. With the exception of copper treatments made just prior to planned bud and shoot removal efforts, the routine use of prophylactic chemical treatments in nurseries does not appear practical, economical or particularly effective.

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